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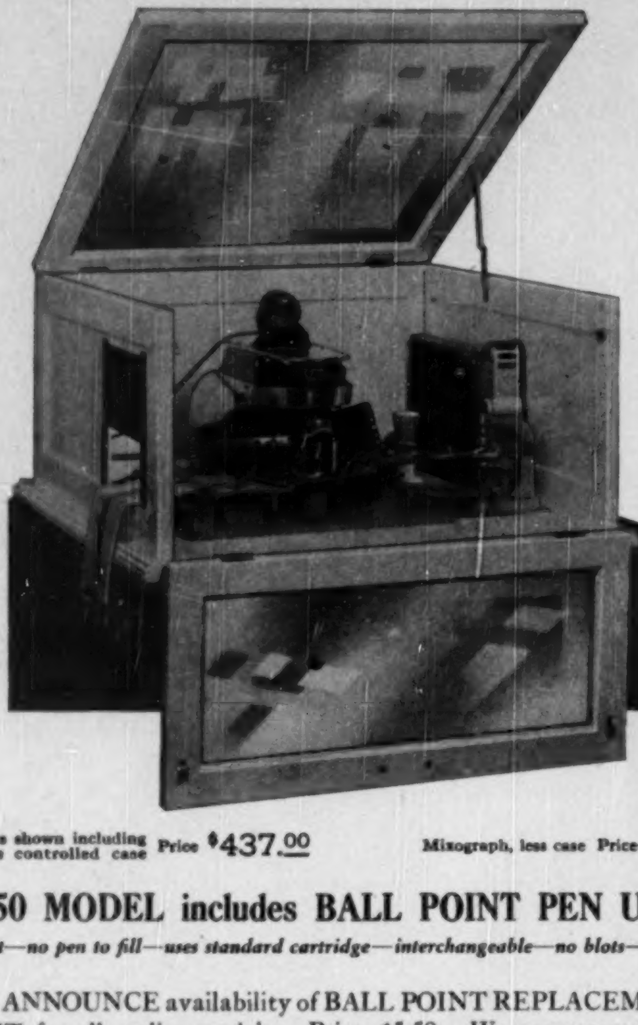

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AN INVESTIGATION OF COMMERCIAL FUNGAL AND BACTERIAL ALPHA-AMYLASE PREPARATIONS IN BAKING[1]

J. F. Conn,[2] J. A. Johnson,[3] and B. S. Miller[3]

ABSTRACT

Commercial alpha-amylase preparations including two bacterial and six fungal sources were employed as diastatic supplements and compared with malted wheat flour. It was demonstrated that fungal preparations may be used for commercial alpha-amylase supplementation if the ratio of proteinase to alpha-amylase is not excessive. The two bacterial preparations caused the bread crumb to be sticky and gummy and were, therefore, undesirable as flour supplements. These properties were associated with the amylase and not the proteinase activity. The reasons for the sticky and gummy bread crumb could not be fully determined but may be explained by the lesser affinity of the bacterial amylase enzyme for low molecular weight dextrins. Separate proteinase and alpha-amylase supplementation studies indicated that hard red spring wheat flours may require more proteinase than hard red winter wheat flours to produce optimum bread. Both alpha-amylase and proteinase supplementation increased the bread crumb compressibility.

The proteinase and alpha-amylase activities of a flour supplemented with a fungal enzyme concentrate decreased during storage at various temperatures. Lower temperatures favored greater retention of enzymatic activity. The rate of decrease was approximately the same whether the supplemented flour was stored in the presence of air, nitrogen, or oxygen. Bacterial spore counts and bread storage data indicated no significant difference in degree of contamination at equivalent levels of supplementation by suitable commercial fungal alpha-amylase preparations and the supplements now in use.

The ability of malted cereals to impart desirable characteristics to bread has been long recognized. In recent decades the desirable characteristics have been shown to result from the action of certain

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Cooperative investigations between the Department of Milling Industry, Kansas State College, Manhattan, Kansas and the Division of Cereal Crops and Diseases, Bureau of Plant Industry, Soils and Agricultural Engineering, Agricultural Research Administration, U. S. Department of Agriculture. Published as Contribution No. 176, Department of Milling Industry, Kansas Agr. Expt. Station.
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enzyme systems which are present in the malted grain. Germinated cereals are the most common source of diastatic supplements. Other possible sources include those produced by culturing bacteria and fungi. There has been extensive commercial production of alpha-amylase by the growth of selected strains of *Bacillus subtilis*, *Bacillus mesentericus*, and *Aspergillus oryzae* on suitable media. The amylases generally produced by these organisms have been found to be of the alpha type. Green (4), Kneen and Sandstedt (10), and Miller and Johnson (13) suggest that fungal alpha-amylase may be suitable for use in baking.

The evaluation of alpha-amylase sources for baking purposes is not a simple problem. Kozmin (11), and Proskuryakov, Grinberg and Kozhevnikova (15) have shown that excessive malt alpha-amylase supplementation caused inelastic and sticky crumb. Miller and Johnson (13), however, observed no stickiness of the crumb when using an aqueous malt extract equivalent to 6% malted wheat flour. Bacterial amylases have shown little promise as diastatic supplements (7) due to the production of sticky bread crumb. Kneen and Sandstedt (10) reported that bacterial amylases have considerably higher thermostability than malt alpha-amylase. Hopkins and Kulka (5) suggested that the differences in the behavior of bacterial and malt alpha-amylase may be due to less affinity of bacterial alpha-amylase for low grade dextrins. Both of these properties could explain the excessive starch liquefaction due to bacterial alpha-amylase resulting in sticky bread crumb characteristics.

Enzymes other than alpha-amylase are also of concern in supplementation. The improvement in loaf volume and grain resulting from the addition of small amounts of several proteolytic preparations to flours was attributed by Read and Haas (16) to a mellowing action on the gluten, giving a more workable dough. Excessive dosages of proteinases have been shown to be detrimental by many workers (3, 7, 9, 13, 16). Miller and Johnson (14) developed technics appropriate for the inactivation of either alpha-amylase or proteinase in malted wheat and barley flour and fungal preparations. Johnson and Miller (9) have studied the role of alpha-amylase and proteinase in bread-making. These workers (9) found that high concentrations of proteinase provided by a fungal preparation caused detrimental effects to loaf volume, grain and texture. Fungal preparations were shown to be satisfactory as diastatic supplements if the ratio of amylase and proteinase is controlled.

While present regulations in the United States permit flour to be supplemented only with malted wheat or barley flour, hearings have been held recently by the Federal Security Agency preparatory to

issuing new "standards of identity" for bread. One subject for consideration has been the use of alpha-amylase from sources other than malted wheat or barley.

The objective of this investigation was to study the characteristics of commercial bacterial and fungal alpha-amylase preparations and to determine the feasibility of their commercial use as alpha-amylase supplements. Consideration also was given to the retention of alpha-amylolytic and proteolytic activity in flours supplemented with a commercial fungal alpha-amylase preparation and stored under controlled conditions.

Materials and Methods

A commercial hard red winter, straight grade flour, having a protein content of 11.8% (14.0% moisture basis), was used for the majority of the experiments. In addition, three hard red spring and two hard red winter bakers' patent flours ranging in protein content from 11.5% to 12.5% were used in studying the separate effects of alpha-amylase and proteinase supplementation. All flours were unmalted and showed good malt response.

The alpha-amylase supplements included a commercial malted wheat flour and eight commercial enzyme concentrates, six of which were of fungal and two of bacterial origin (Table I).

TABLE I

Alpha-Amylase and Proteinase Activities of Various Enzyme Preparations Compared to Malted Wheat Flour

Enzyme	Ratios of activities		
	Alpha-amylase	Proteinase	Ratio of proteinase to alpha-amylase
Malted wheat flour[1]	1	1	1
Maltase-20 (fungal)	66	67	1
Rhozyme-S (fungal)	120	1000	8.3
Diastase-29 (fungal)	0.21	417	2000
Diastase-32 (fungal)	5.7	244	43
Diastase-33 (fungal)	50	31.6	0.63
Diastase-34 (fungal)	80	0.19	0.002
Diastase-28 (bacterial)	31	5.7	0.18
Diastase-30 (bacterial)	24	69	2.9

[1] 40 alpha-amylase units of activity (17) per gram.

The sponge-dough baking procedure reported by Johnson and Miller (9) was employed. Alpha-amylase dextrinization activity was determined by the procedure described by Sandstedt, Kneen and Blish (17).[4] The starch liquefying activity of the alpha-amylase was de-

[4] Although the method used for determining dextrinogenic activity was designed for malt preparations, it has also been used in this study for determining the dextrinogenic activity of both fungal and bacterial amylases.

termined with the amylograph employing 65 gms. of flour and 450 ml. of liquid as described by Anker and Geddes (2). Proteinase activity was determined by the Ayre-Anderson method as modified by Miller (12).

Arbitrary terms were defined to indicate the proteinase and alpha-amylase concentrations used in baking. The term "1X" indicates alpha-amylase supplementation equivalent to the alpha-amylase added by 0.25% malted wheat flour having an activity of 40 alpha-amylase units per gm. The term "1Y" indicates a level of proteinase supplementation per 100 g. of flour equal to that amount of proteinase which would give a titration of one ml. of 0.0714 *N* sodium hydroxide in the proteolytic activity determination (12).

The effect of temperature on the inactivation of alpha-amylase from various sources was determined by the technique used by Johnson and Miller (8). Techniques used for the differential inactivation of alpha-amylase and proteinase were described by Miller and Johnson (14).

Crumb compressibility was determined with a Bloom Gelometer after storage of the bread in sealed plastic bags for periods of 24 and 96 hours. The experimental value was expressed in grams of lead shot required to press a one-inch plunger 4 mm. into a slice of bread. Two determinations on each of two slices cut from three loaves chosen from each experimental group were recorded.

The effect of oxygen, nitrogen, and air atmospheres on enzyme activity was studied by storing flour supplemented with Rhozyme-S, one of the fungal preparations, at a level equivalent to 1% malted wheat flour alpha-amylase. The samples were stored at 5°C., room temperature, and 35°C. for eight months.

The A.A.C.C. method (1) for determining the total bacterial spores in flour was used for determination of spore counts in the various enzyme preparations. James and Smith (6) have indicated that this method gives only presumptive indications of the number of rope spores.

Results and Discussion

The alpha-amylase activities of six fungal and two bacterial preparations were found to vary from a fraction to as much as 120 times that of the commercial malted wheat flour (Table I). The proteinase activities of these same enzyme concentrates were found to vary from a fraction to as much as 1,000 times that of malted wheat flour. The ratio of proteinase to alpha-amylase was found to vary from a fraction to as much as 2,000 times that of malted wheat flour.

Preliminary experimental sponge baking studies using quantities of these enzyme preparations which provided equivalent alpha-

amylase levels indicated that the bacterial preparations, Diastase-28 and -30, produced sticky bread crumb. These preparations produced no evidence of excessive proteolytic activity. Diastase-29 and Diastase-32, however, contained excessive amounts of proteinase as measured by chemical means and as indicated during fermentation. No sticky bread crumb was observed in bread containing these preparations. Rhozyme-S, Maltase-20, Diastase-33, and Diastase-34, as well as the two bacterial preparations were believed to warrant further investigation.

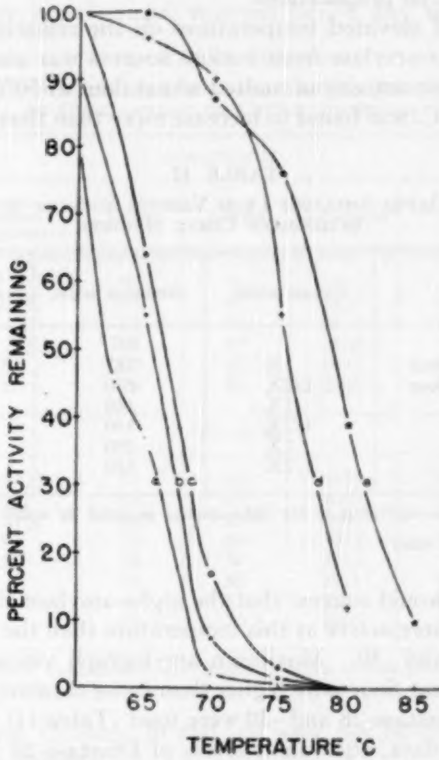

Fig. 1. The effect of heating and enzyme source on the retention of alpha-amylase activity; a, Diastase-28, bacterial; b, Diastase-30, bacterial; c, Rhozyme-S, fungal; d, Malted wheat flour; and e, Rhozyme-DX, bacterial.

Bacterial Concentrates as Enzyme Supplements. The evidence in the literature (7, 10) suggests that some bacterial alpha-amylases have high thermostability. The effect of temperature on the inactivation of alpha-amylase was studied, therefore, as one of the possible explanations of crumb stickiness resulting from the use of bacterial Diastase-28

and -30. The results (Fig. 1) obtained for various preparations including those for a bacterial preparation (Rhozyme-DX) known to possess high thermostability show that the alpha-amylases of Diastase-28 and -30 were actually less thermostable than malted wheat flour alpha-amylase. Rhozyme-DX, as expected, possessed high thermostability. These results showing that amylases from different bacterial strains may differ in thermostability corroborate the work of Tilden and Hudson (18). Thus, the thermostability of these bacterial alpha-amylases does not explain the reason for sticky crumb caused by these particular bacterial preparations.

The effect of elevated temperatures on the relative dextrinogenic activity of alpha-amylase from various sources was also investigated. The dextrinogenic activity of malted wheat flour at 50°C. as compared with that at 30°C. was found to increase more than that of Diastase-28

TABLE II

EFFECT OF ALPHA-AMYLASES FROM VARIOUS SOURCES ON MAXIMUM AMYLOGRAPH CURVE HEIGHTS

Preparation	Concentration[1]	Maximum height	Per cent height of corresponding malted wheat flour curve
		B.U.[2]	%
Malted wheat flour	1X	385	—
Malted wheat flour	1/2X	630	—
Diastase-28	1X	340	88
Diastase-28	1/2X	550	87
Diastase-30	1X	280	73
Diastase-30	1/2X	480	76

[1] 1X—concentration equivalent to the alpha-amylase provided by 0.25% malted wheat flour supplementation.
[2] B.U.—Brabender units.

and -30. This would suggest that the alpha-amylase of malted wheat flour would be more active at this temperature than the alpha-amylase of Diastase-28 and -30. Maximum amylograph viscosities obtained with malted wheat flour were higher than those obtained when equivalent levels of Diastase-28 and -30 were used (Table II). Thus, based on amylograph data, equivalent levels of Diastase-28 and -30 would be expected to produce greater starch degradation than an equivalent level of malted wheat flour. However, from the temperature of inactivation data and the dextrinization activities at the elevated temperature, malted wheat flour would be expected to produce greater starch degradation than the bacterial preparations. This apparent anomaly may be explained by assuming a lesser affinity of bacterial alpha-amylase for lower molecular weight dextrins as suggested by Hopkins and Kulka (5). Accordingly bacterial alpha-amylase mole-

cules may be free to split greater numbers of starch molecules, with a corresponding increase in dextrin formation and stickiness of bread crumb.

Crumb stickiness due to the presence of bacterial alpha-amylase was evident at 0.1X concentration of either Diastase-28 or -30. This stickiness was accentuated at higher concentrations. No dough stickiness associated with excessive proteolytic activity was observed at any time during fermentation. From the baking results (Table III) it was concluded that as the concentration of Diastase-28 or -30 was increased, sticky bread crumb resulted before any beneficial effects

TABLE III

EFFECT OF STARCH LIQUEFYING AND SACCHARIFYING ENZYMES ON DEVELOPMENT OF CRUMB STICKINESS

Rhozyme-S concentration[1]	Bacterial enzyme concentration[1]	Grain	Texture	Loaf volume	Crumb properties
DIASTASE-28					
		%	%	cc.	
0	0.04X	68	70	2835	Satisfactory
0	0.1X	65	65	2840	Slightly sticky
0	0.25X	67	65	2830	Slightly sticky
1X	1X	50	50	3015	Very sticky
4X	1X	55	50	2940	Very sticky
DIASTASE-30					
0	0.04X	65	65	2950	Satisfactory
0	0.1X	65	65	3060	Slightly sticky
0	0.25X	60	65	2900	Slightly sticky
1X	1X	55	50	3025	Very sticky
4X	1X	50	50	2915	Very sticky
1X	0	85	80	2975	Satisfactory
4X	0	80	83	2925	Satisfactory
0	0	70	75	2865	Satisfactory

[1] 1X—concentration of alpha-amylase equivalent to the alpha-amylase provided by 0.25% malted wheat flour supplementation.

appeared. Thus, Diastase-28 and -30 were not suitable as diastatic supplements for baking.

Fungal Concentrates as Enzyme Supplements. An investigation was made of supplementation with Rhozyme-S, Maltase-20, Diastase-33 and -34 compared with malted wheat flour using alpha-amylase concentrations of 1X, 4X, and 8X. The results are shown in Table IV. Each fungal preparation was blended with flour to give a product equivalent in alpha-amylase activity to that of malted wheat flour. Optimum results were obtained with malted wheat flour at 4X concentration while Rhozyme-S and Maltase-20 produced best results at

TABLE IV

BAKING DATA USING RHOZYME-S AND MALTASE-20, DILUTED WITH FLOUR AS ALPHA-AMYLASE SUPPLEMENTS

Preparation	Concentration[1]	Grain	Texture	Loaf volume	Dough properties
		%	%	cc.	
———	0	80	80	2858	Satisfactory
Malted wheat flour	1X	90	90	2971	Satisfactory
Malted wheat flour	4X	92	90	3025	Slightly slack
Malted wheat flour	8X	85	85	3105	Slightly slack
Rhozyme-S	1X	87	85	2992	Slightly slack
Rhozyme-S	4X	80	83	2963	Slack
Rhozyme-S	8X	70	80	2945	Very slack
Maltase-20	1X	90	88	2942	Satisfactory
Maltase-20	4X	88	88	3058	Slightly slack
Maltase-20	8X	70	75	2975	Slightly slack
Diastase-33	1X	88	88	2947	Satisfactory
Diastase-33	8X	90	90	2920	Slightly slack
Diastase-34	1X	88	90	3020	Satisfactory
Diastase-34	8X	92	92	3060	Satisfactory

[1] 1X—concentration equivalent to the alpha-amylase provided by 0.25% malted wheat flour supplementation.

1X concentration. The bread baked with Diastase-33 and -34 did not exhibit a marked optimum alpha-amylase level, but appeared to be adequately supplemented at 1X concentration. Increasing the concentration of Rhozyme-S and Maltase-20 beyond the 1X level caused the grain and texture to become inferior. There was no evidence of sticky or gummy crumb characteristics at these high alpha-amylase concentrations. The dough, however, slackened excessively during

TABLE V

BAKING DATA USING RHOZYME-S WITH VARIOUS PERCENTAGES OF THE PROTEINASE REMOVED

Alpha-amylase concentration[1]	Proteinase retained	Grain	Texture	Loaf volume	Dough properties
	%	%	%	cc.	
0	—	70	70	2833	Satisfactory
1X	10	85	90	2916	Satisfactory
1X	25	75	85	2796	Satisfactory
1X	50	80	80	2696	Slightly slack
1X	75	75	75	2879	Slack
4X	10	85	90	3008	Satisfactory
4X	25	83	88	2971	Satisfactory
4X	50	78	85	2946	Slack
4X	75	75	75	2879	Slack

[1] 1X—concentration equivalent to the alpha-amylase provided by 0.25% malted wheat flour.

fermentation due to excessive proteolysis and became difficult to machine when the highest enzyme concentrations were employed.

Baking results obtained with Rhozyme-S from which various proportions of the total proteinase activity had been removed are presented in Table V. A tendency to produce slack dough was noted when 50% of the proteinase was present and considerable slackening was observed at both 1X and 4X concentrations of alpha-amylase containing three-fourths of the original proteinase activity. The best

TABLE VI

Baking Data Indicating the Separate Effects of Alpha-Amylase and Proteinase Supplementation

Rhozyme-S alpha-amylase concentration[1]	Diastase-29 proteinase concentration[2]	Grain	Texture	Loaf volume	Dough properties
HARD RED WINTER FLOUR					
		%	%	cc.	
0	0	75	80	2916	Satisfactory
0	1Y	80	78	2981	Satisfactory
0	4Y	85	80	2988	Slightly slack
1X	0	90	88	3059	Satisfactory
1X	1Y	90	88	3041	Slightly slack
1X	4Y	88	85	2959	Slack
8X	0	90	90	3062	Satisfactory
8X	1Y	88	90	3066	Slightly slack
8X	4Y	85	87	3056	Slack
HARD RED SPRING FLOUR					
0	0	75	80	2788	Slightly bucky
0	1Y	77	82	2738	Satisfactory
0	4Y	77	82	2869	Satisfactory
1X	0	80	85	2747	Satisfactory
1X	1Y	85	87	2975	Satisfactory
1X	4Y	92	90	2994	Slightly slack
8X	0	80	87	2888	Satisfactory
8X	1Y	92	90	2953	Slightly slack
8X	4Y	92	90	2959	Slightly slack

[1] 1X—concentration of alpha-amylase equivalent to the alpha-amylase provided by 0.25% malted wheat flour supplementation.
[2] 1Y—the addition to 100 grams of flour of that amount of Diastase-29 proteinase which would give a titration of one ml. in a proteolytic activity determination.

bread was obtained when only 10% to 25% of the original proteinase was present. These results demonstrated the desirability of reducing the proteinase activity of Rhozyme-S.

The data in Table V were further substantiated by studies in which the alpha-amylase and proteinase were added as separate adjuncts. Rhozyme-S having up to 98% of the proteinase activity removed was used in combination with small amounts of the proteinase derived from

the same preparation by appropriate differential inactivation techniques. Both external and internal characteristics indicated that 4X and 8X concentration of the alpha-amylase provided optimum amylase supplementation for a hard red winter wheat flour. Some improvement in quality of the bread was observed for proteinase supplementation equivalent to that provided by 1X concentration of the original preparation.

TABLE VII

EFFECT OF ENZYME SOURCE ON BREAD CRUMB COMPRESSIBILITY

Enzyme source	Concentration	Compressibility of bread stored[1]	
		24 hours	96 hours
		g.	g.
Control	0	98.8	126.8
Malted wheat flour	X	89.6	113.1
Malted wheat flour	4X	94.5	98.3
Rhozyme-S	X	92.7	94.8
Rhozyme-S	4X	79.4	100.4
Maltase-20	X	84.5	88.7
Maltase-20	4X	79.0	85.8

ANALYSIS OF VARIANCE

Source of variation	Degrees of freedom	Mean square
Enzyme concentration	1	1290**
Days of storage	1	5100***
Enzyme source	2	1717***
Concentration × storage	1	142
Concentration × source	2	279*
Storage × source	2	525*
Conc. × storage × source	2	613**
Within source	132	120

[1] Average of twelve separate readings. The grams of lead shot required to press a one-inch plunger 4 mm. into bread crumb one inch thick.

* Significance exceeds 5% level.

** Significance exceeds 1% level.

*** Significance exceeds 0.1% level.

$\bar{x}_{.01}$ = 11.68—the difference between compressibility values for different enzyme sources required for significance at the 1% level.

$\bar{x}_{.05}$ = 8.84—the difference between compressibility values for different enzyme sources required for significance at the 5% level.

Further investigations concerning the separate effects of alpha-amylase and proteinase were performed using Diastase-29, proteinase, and Rhozyme-S from which the proteinase had been removed as the source of alpha-amylase. Baking experiments were performed using three commercially milled hard red winter and three commercially milled hard red spring wheat flours with various combinations of these enzymes. Typical results are shown in Table VI. Optimum bread for the hard red winter flour was obtained when no proteinase was

added but the flour easily tolerated proteinase up to 1Y concentration. It was concluded that optimum quality bread was obtained when the hard red winter wheat flours under investigation received 0 to 0.5Y proteinase supplementation.

Optimum quality bread was obtained with the hard red spring wheat flour when 1 to 4Y concentration of Diastase-29 was used. The results obtained for two additional flours also indicated that at least 1Y concentration of Diastase-29 was required for optimum proteinase supplementation. There appeared to be little difference between 1X and 8X alpha-amylase supplementation. The results obtained indicate that the optimum level of proteinase supplementation for hard red spring wheat flours is substantially greater than the optimum level for hard red winter wheat flours.

TABLE VIII

EFFECT OF FUNGAL ALPHA-AMYLASE AND PROTEINASE SUPPLEMENTATION ON BREAD CRUMB COMPRESSIBILITY

Alpha-amylase concentration[1]	Proteinase concentration[2]	Compressibility of bread stored	
		24 hours	96 hours
		g.[3]	g.[3]
0	0	100.4	152.0
0	1Y	95.1	149.3
0	4Y	85.8	144.4
1X	0	83.9	131.6
1X	1Y	82.3	125.3
1X	4Y	74.6	120.5
8X	0	82.2	126.6
8X	1Y	75.8	120.4
8X	4Y	71.0	118.7

[1] 1X—concentration of alpha-amylase equivalent to the alpha-amylase provided by 0.25% malted wheat flour supplementation.
[2] 1Y—concentration, the addition to 100 grams of flour of that amount of Diastase-29 proteinase which would give a delta titration of 1 ml. in the proteolytic activity determination.
[3] The grams of lead shot required to press a one-inch plunger 4 mm. into bread crumb, one inch thick. Average of twelve separate readings.

A comparison of the effects of malted wheat flour, Rhozyme-S and Maltase-20 on the compressibility of bread is shown in Table VII. The analysis of variance for this data also is included in Table VII. Enzyme source and concentration, as well as length of storage, affected the compressibility significantly. After 24 hours of storage, the bread baked with 4X concentration of Rhozyme-S, 1X and 4X concentration of Maltase-20 was significantly more compressible than bread containing no enzyme supplement. After 96 hours of storage, all enzyme treatments produced bread which was significantly more compressible than the control bread. At X concentration of alpha-amylase, bread baked with either Rhozyme-S or Maltase-20 was significantly more

compressible than the bread baked with a corresponding concentration of alpha-amylase from malted wheat flour.

The separate effects of alpha-amylase and proteinase supplementation on bread crumb compressibility are illustrated in Table VIII. Data were obtained after 24 and 96 hours of storage. The analysis of variance of these data is shown in Table IX. Both alpha-amylase and proteinase increased, while storage decreased the crumb compressibility. From calculations of least significant mean differences, it was determined that 4Y concentration of proteinase was required to produce a crumb compressibility significantly greater than that of the crumb of bread containing no added proteinase. The bread crumb of loaves baked with either 1X or 8X concentration of alpha-amylase was significantly more compressible than the crumb of loaves containing no added alpha-amylase. No significant differences in compressibility,

TABLE IX

ANALYSIS OF VARIANCE OF THE SEPARATE EFFECT OF ALPHA-AMYLASE, PROTEINASE, AND STORAGE ON BREAD CRUMB COMPRESSIBILITY

Source of variation	Degrees of freedom	Mean square
Proteinase	2	1,926**
Alpha-amylase	2	9,988**
Storage	1	127,653**
Proteinase × alpha-amylase	4	26
Proteinase × storage	2	57
Alpha-amylase × storage	2	517**
Proteinase × alpha-amylase × storage	4	35
Individual values	198	119
Total	215	

** Significant at the 1% level.

however, were observed between 1X and 8X alpha-amylase concentration. Combinations of alpha-amylase and proteinase produced bread crumb with greater compressibility. It would appear that the choice of alpha-amylase level might not be so critical as that of the proteinase level.

Stability During Storage of Proteinase and Alpha-Amylase in Rhozyme-S Supplemented Flour. The effects of storage on the retention of alpha-amylase and proteinase activity of flours stored under oxygen, nitrogen, and air atmosphere at three different temperatures are shown in Fig. 2. The analyses of variance of these data are presented in Table X. Significant differences in both alpha-amylase and proteinase as affected by temperature and length of storage were observed. The lower storage temperature was favorable to the retention of both alpha-amylase and proteinase. The effect of storage under various gases was similar.

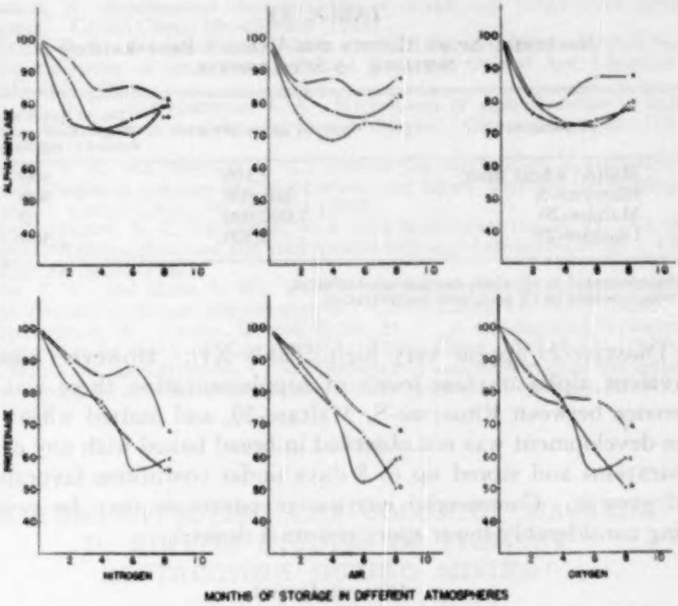

Fig. 2. The effect of gaseous atmosphere, temperature and length of storage on the retention of alpha-amylase and proteinase activities in flour supplemented with Rhozyme-S; a, 5°C; b, room temperature; and c, 35°C.

Microflora of Enzyme Preparations. The number of bacterial spores per gram of preparation was determined for various enzyme concentrates suitable for supplementation. The counts for Rhozyme-S

TABLE X

Analyses of Variance of the Effect of Temperature and Gaseous Atmosphere upon the Retention of Alpha-Amylase and Proteinase Activity During Storage

Source of variation	Degrees of freedom	Mean square	
		Alpha-amylase activities	Proteinase activities
Atmosphere	2	5	19
Temperature	2	315**	413**
Length of storage	4	857**	2752**
Atmosphere × temperature	4	7	9
Atmosphere × length of storage	8	10	6
Temperature × length of storage	8	29	76**
Atmosphere × length of storage × temperature	16	8	13
Total	44		

** Significant at the 1% level.

TABLE XI

BACTERIAL SPORE COUNTS FOR VARIOUS PREPARATIONS SUITABLE AS SUPPLEMENTS

Preparation	Bacterial spores per gram	No. of spores added to 100 g. of flour when supplemented to equivalent levels
Malted wheat flour	2,100	500[1]
Rhozyme-S	405,000	800[1]
Maltase-20	1,000,000	10[1]
Diastase-29	2,500	2,300[2]

[1] Supplemented to 1X alpha-amylase concentration.
[2] Supplemented to 1Y proteinase concentration.

and Diastase-29 appear very high (Table XI). However, based on equivalent alpha-amylase levels of supplementation there was little difference between Rhozyme-S, Maltase-20, and malted wheat flour. Rope development was not observed in bread baked with any of these preparations and stored up to 8 days under conditions favorable for mold growth. Commercial enzyme preparations may be prepared having considerably lower spore counts if desired.

Acknowledgment

The financial assistance provided by Rohm and Haas Company, Philadelphia, is gratefully acknowledged. Acknowledgment is also made the Rohm and Haas Company for the numerous enzyme concentrates made available for this study and to the Kansas Milling Company, Wichita, Kansas, for the malted wheat flour.

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FACTORS AFFECTING THE COLOR OF MACARONI
II. KINETIC STUDIES OF PIGMENT DESTRUCTION DURING MIXING [1]

G. N. IRVINE [2] and C. A. WINKLER [3]

ABSTRACT

The destruction of the xanthophyll pigments during the mixing stage of macaroni processing has been followed kinetically under a variety of mixing conditions. The rate increases with increasing absorption to a maximum at about 33%; with increasing temperature; and with increasing oxygen concentration of the mixing atmosphere. The reaction is inhibited by thousandth molar cyanide and alpha naphthol and by alcohol concentrations above 10%. Reversal of the reaction occurs on prolonged mixing in 40% alcohol. Evidence is provided to support the hypothesis that the destruction of pigment occurs through a coupled reaction involving the peroxidation of unsaturated fat by the enzyme lipoxidase. It is proposed that this enzyme system functions, in semolina, in conjunction with an activating enzyme which is inhibited by cyanide and accelerated by some heavy metal ions. A mechanism is proposed for the reaction which accounts for the phenomena observed.

Most varieties of durum wheat grown in North America yield macaroni which is physically satisfactory, handles well, and has good

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[3] Professor of Chemistry, McGill University.

cooking quality. Hence the differentiation of good and poor varieties for macaroni-making purposes is made largely on the basis of the color of the macaroni made therefrom. This should be a bright yellow or yellow amber color.

Macaroni with a high yellow pigment content can be obtained only from wheat that is high in this pigment, and plant breeders are constantly striving to produce such wheats. This factor in itself, however, does not necessarily ensure a good yellow macaroni. There are several older varieties, such as Golden Ball and Pelissier, which have a characteristically high yellow pigment content, but which produce macaroni that loses a great deal of pigment during processing. It has been found in this laboratory that the percentage of pigment destroyed during processing varies from about 20% for the best varieties to about 60% for the poorest varieties. Destruction has been shown to occur principally during mixing, though a small additional loss occurs during extrusion. This paper describes a kinetic study, dealing with the decrease in pigment with time under a variety of mixing conditions, that serves to elucidate the principal factors and mechanisms involved in destruction of the yellow pigments.

Materials and Methods

Samples of a number of pure varieties covering a wide range of macaroni-making quality were used in the investigation. Semolinas were milled to 50% yield on an Allis-Chalmers laboratory mill.

The semolinas were processed by the disc method of Cunningham and Anderson (5) which involves preparing doughs from 50 g. samples in a micro mixer. This mixer, because of its small scale and special design, is much more severe than commercial mixers; it produces a cohesive and relatively uniform dough in about 1¼ minutes, as compared with 10 to 15 minutes for commercial models. Thus mixing times reported in this paper are not directly comparable with those obtained in commercial plants.

Studies with the micro macaroni method (8) have shown that 31% absorption is preferable to the 30% used in the disc method, and the former level was therefore used in this study. Since results can be reproduced within 2 or 3%, the disc method is ideally suited to a kinetic study. In addition, it has been shown (1) that, with this method, pigment losses occur almost entirely during mixing.

The dried discs were ground in a semi-micro Wiley Mill to pass a 60-mesh sieve. Pigment was extracted with water-saturated butyl alcohol and determined in an Evelyn Colorimeter. All the pigment determinations were corrected to a 14% moisture basis.

Results and Discussion

Preliminary experiments were made with a number of good and poor varieties to gain some knowledge of the characteristics of the reaction. Mixing was carried out at 30°C. in an atmosphere of air for intervals up to 10 mins. Typical curves for an excellent and a very poor variety are shown in Fig. 1. Other varieties were found to yield a similar type of curve with rates falling somewhere between these two. Fig. 1 indicates that the reaction occurs in three distinct

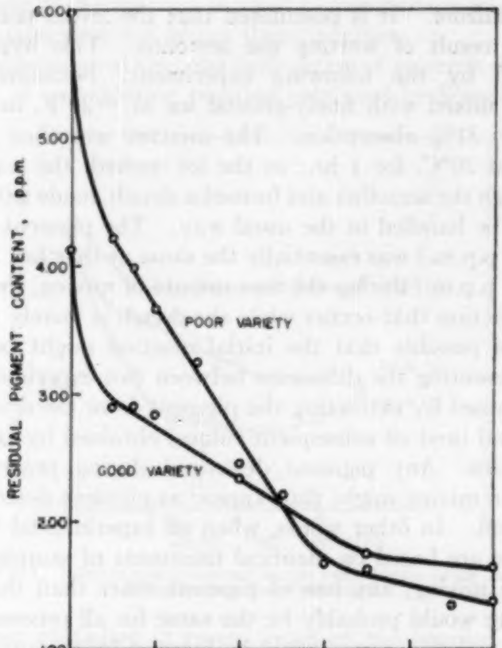

Fig. 1. Typical reaction curves for pigment loss during mixing of doughs representing a good and a poor variety of durum wheat.

stages: (1) a rapid reaction during the first minute of mixing; (2) a slower zero order reaction during the period from 1 to about 6 mins. of mixing; and (3) a still slower reaction, apparently zero order, beyond six minutes of mixing. The discussion in this paper deals only with the first two stages as it is unlikely that the third stage is reached during commercial processing of macaroni.

For convenience, the first two stages of the reaction will be called the "initial reaction" and the "mixing reaction" respectively. A distinction seems logical because the condition of the dough changes

after about 1 min. of mixing. At first, the revolving pins merely tumble the lumps of wet semolina through the fixed pins of the mixing trough. After about 1 min., a cohesive dough forms rapidly and the mixer does work on the dough through the shearing action that occurs as the revolving pins force the cohesive dough between the fixed pins.

Fig. 1 shows that the rates of both the initial reaction and the mixing reaction are much greater for the poor variety than for the good variety.

Initial Reaction. It is postulated that the initial reaction occurs merely as a result of wetting the semolina. This hypothesis was substantiated by the following experiment. Semolina cooled to $-20°$F. was mixed with finely-ground ice at $-20°$F. in an amount equivalent to 31% absorption. The mixture was then placed in a water bath at 30°C. for 1 hr.; as the ice melted, the water diffused evenly through the semolina and formed a dough, made *without mixing*, which could be handled in the usual way. The pigment lost by this sample (0.65 p.p.m.) was essentially the same as that lost by a control sample (0.62 p.p.m.) during the first minute of mixing, that is, during the initial reaction that occurs while the dough is merely tumbled.

It seemed possible that the initial reaction might be merely an artifact representing the difference between two experimental values; the first obtained by extracting the pigment from the dried semolina, and the second (and all subsequent values) obtained by extracting the processed discs. Any pigment destroyed during processing stages subsequent to mixing might thus appear as pigment destroyed by the initial reaction. In other words, when all experimental values other than the first are based on identical treatment of samples except for variations in mixing, any loss of pigment other than that occurring during mixing would probably be the same for all processed discs; as a result, the reaction curve would be lowered by a constant amount, and this might be interpreted to indicate an initial reaction which would then be an artifact rather than a reality.

To investigate this possibility, a sample was mixed and immediately extracted in the wet with a Waring Blendor. The pigment lost by this sample (2.20 p.p.m.) was essentially the same as that lost (2.23 p.p.m.) when a corresponding sample was processed into discs and dried. It was thus shown that the initial reaction actually does take place in the interval from zero time to 1 min. of mixing. Further support for this hypothesis was obtained in experiments on inhibition described in a later subsection.

Mixing Reaction. The foregoing experiment emphasizes a very interesting characteristic of the mixing reaction. Destruction of

pigment obviously occurs during mixing. When mixing is stopped, the destruction of pigment ceases even though the dough is handled wet through several subsequent stages and is finally dried over a period of 48 hrs. The explanation appears to depend on the emulsifying action of the mixer. The pigment, being fat soluble, is probably associated in the cell with the lipid fraction. After the addition of water and the formation of a cohesive dough, the lipid and aqueous phases are apparently emulsified by mixing thus enabling the reaction to proceed. It is also suggested that when the two phases are combined, the rate of the reaction is rapid by comparison with the rate at which the mixing reaction brings them together.

It has been assumed that the destruction of pigment during mixing is the result of an oxidation reaction catalysed by lipoxidase. Much

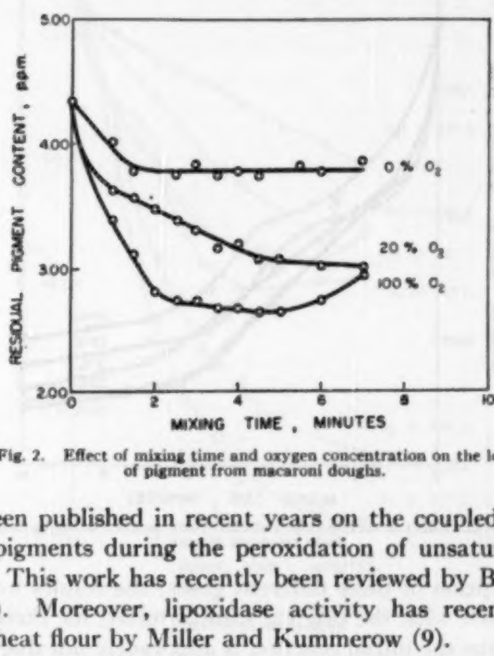

Fig. 2. Effect of mixing time and oxygen concentration on the loss of pigment from macaroni doughs.

work has been published in recent years on the coupled oxidation of carotenoid pigments during the peroxidation of unsaturated fats by lipoxidase. This work has recently been reviewed by Bergström and Holman (3). Moreover, lipoxidase activity has recently been reported in wheat flour by Miller and Kummerow (9).

Oxygen Concentration. Several experiments were made to determine the effect of oxygen concentration on the reaction. Samples were mixed at 30°C. in atmospheres of nitrogen, air, or oxygen. The results of one such experiment are shown in Fig. 2. The most interesting feature of this experiment is the course of the reaction under an atmosphere of nitrogen (curve labeled, 0% O_2). The "initial reaction" takes place, but no further reaction occurs. Thus the mixing action produces no destruction of pigment in the absence of an atmosphere

containing oxygen. From a comparison of the reaction in air and in oxygen it is possible that the rates of both the initial and the mixing reactions increase with increasing oxygen concentration.

This experiment suggested that the initial reaction might be due to oxygen adsorbed on the semolina particles. Three samples were evacuated for three minutes and then nitrogen, air, and oxygen, respectively, were introduced. Each sample was then mixed under an atmosphere of nitrogen for three minutes to isolate the initial reaction. It was assumed that the evacuation would remove any adsorbed gases and that the nitrogen, air, or oxygen would subsequently be adsorbed on the semolina particles. No difference was found as a result of the

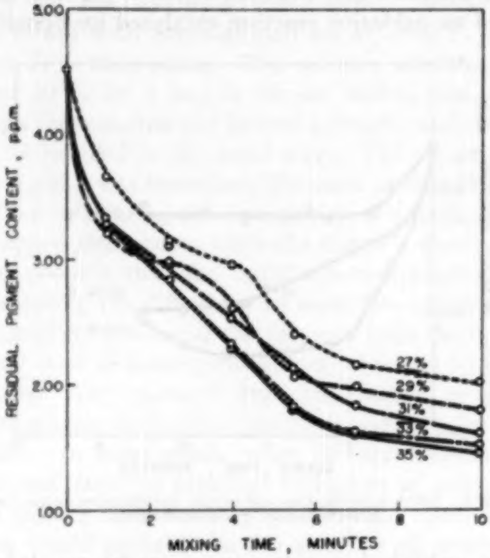

Fig. 3. Effect of mixing time and absorption on the loss of pigment from macaroni doughs.

possible adsorption of these different gases; the results were the same as that obtained with the original sample mixed for three minutes in nitrogen. Thus the initial reaction is apparently not due to adsorbed oxygen and must result from a more strongly held oxygen complex in the semolina—possibly within the unruptured cells of the original semolina particles.

Absorption. The effect of absorption was studied at five levels: 27%, 29%, 31%, 33%, and 35%. The results of the experiment are shown in Fig. 3. In general, the rate of the initial reaction is slower at lower absorptions, and there appears to be an induction period before the mixing reaction commences. The latter effect at low

absorptions probably results from slower formation of the cohesive dough that has been postulated as a necessary condition for the mixing reaction to proceed. There appears to be a limiting value for the absorption beyond which no increase in rate is apparent. This value was 33% for the particular sample used, but may vary from sample to sample as it is probably connected with the quantity and quality of the protein present.

Temperature. The effect of temperature on the reaction in air and in oxygen is shown in Fig. 4. It is evident that the temperature effect

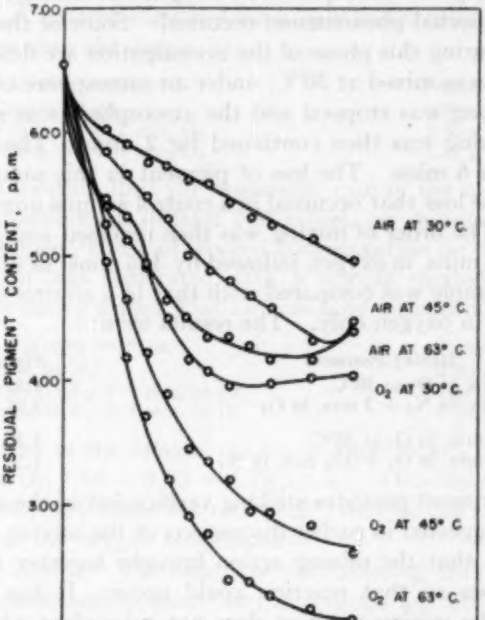

Fig. 4. Effect of mixing time and temperature on the loss of pigment from macaroni doughs.

is complex. The rate of the initial reaction increases with temperature in both air and oxygen and the rate of the mixing reaction increases as well. Because of the interaction of many factors, an interpretation of these results is difficult. Increasing temperature will hasten the formation of a cohesive dough. It will also affect the state of the dough and the rate of emulsification of the enzyme and lipid phases. In addition, it will displace any chemical equilibria involved in the reaction. It is interesting to note that the rate of the mixing reaction

is constant during some period at each temperature in air, while this is not true of any of the doughs mixed in oxygen. It is possible that when mixing is done in air, the partial pressure of oxygen is a limiting factor.

Mixing in Nitrogen and Oxygen. In the course of some auxiliary experiments on the effect of oxygen at higher temperatures, an interesting effect was observed which seemed to have a direct bearing on the mechanics of the mixing reaction. It was found that if mixing were carried on for some time under an atmosphere of nitrogen, and then this atmosphere was replaced by oxygen and mixing recommenced, a rather unexpected phenomenon occurred. Some of the experiments undertaken during this phase of the investigation are described below.

A sample was mixed at 30°C. under an atmosphere of nitrogen for 4 mins. Mixing was stopped and the atmosphere was replaced with oxygen. Mixing was then continued for 2 mins. The total mixing time was thus 6 mins. The loss of pigment in this sample was compared with the loss that occurred in a control sample mixed for 6 mins. in oxygen. The order of mixing was then reversed and a sample was mixed for 1½ mins. in oxygen followed by 3½ mins. in nitrogen. The loss in this sample was compared with that in a control sample mixed for 1½ mins. in oxygen only. The results were:

	Mixing treatment	*Pigment loss*
1.	6 min. in O_2 at 30°C.	2.52 p.p.m.
	4 min. in N_2 + 2 min. in O_2	2.77 p.p.m.
2.	1½ min. in O_2 at 30°C.	1.20 p.p.m.
	1½ min. in O_2 + 3½ min. in N_2	1.18 p.p.m.

This experiment provides striking verification of the emulsification hypothesis suggested in earlier discussions of the mixing reaction. It was proposed that the mixing action brought together the lipid and aqueous phases so that reaction could occur. It has been shown earlier that the mixing reaction does not take place when mixing is carried out under nitrogen. Yet a sample mixed for 4 mins. in nitrogen, then 2 mins. in oxygen, loses a similar amount of pigment to that of a sample mixed for the whole 6 mins. in oxygen. Hence it may be concluded that the two phases are brought in contact during mixing, but that no reaction can occur beyond the initial reaction until oxygen is introduced; once oxygen is introduced and mixing recommenced, the potential reaction which has been built up in the system by the mixing action can occur rapidly.

The second part of this experiment shows that the means by which oxygen comes into contact with the system is crucial to the progress of the reaction. If the dough is mixed for a short time in oxygen after a longer mixing period in nitrogen, the amount of pigment destroyed

depends on the oxygen concentration and on the *total* mixing time; but, if mixing is first done in oxygen, and nitrogen is then introduced, the reaction is immediately stopped. The partial pressure of the residual oxygen in the dough under an atmosphere of nitrogen will be small, and it thus appears that a certain minimum partial pressure of oxygen is necessary to bring about the pigment oxidation. This is one of the essential steps of the reaction.

Information on the rate of reaction in an atmosphere of oxygen after a preliminary treatment of 4 mins. mixing in nitrogen was sought by using a shorter mixing time in oxygen than that of the previous experiment. A control was run 4 mins. only in nitrogen. The following pigment losses were obtained:

Mixing treatment	*Pigment loss*
1. Control, 4 min. N_2	0.80 p.p.m.
2. 4 min. N_2 + 1 min. O_2	2.56 p.p.m.
3. 4 min. N_2 + 2 min. O_2	2.77 p.p.m.

These results indicate that the maximum rate of the mixing reaction under these conditions is at least 2.56 p.p.m. per minute.

The pronounced effect of oxygen concentration on this "delayed" mixing reaction was illustrated with a sample treated in a similar manner to the above except that air was used instead of oxygen:

Mixing treatment	*Pigment loss*
1. 4 min. in N_2	0.80 p.p.m.
2. 4 min. in N_2 + 1 min. in air	1.21 p.p.m.
3. 4 min. in N_2 + 1 min. in O_2	2.56 p.p.m.

The net loss due to the *delayed* mixing is then: in air, $1.21 - 0.80 = 0.41$ p.p.m.; in O_2, $2.56 - 0.80 = 1.76$ p.p.m. Thus the rate in oxygen is approximately 4½ times the rate in air; that is, the rate is roughly proportional to the oxygen concentration.

Inhibiting Agents. The effects of the following enzyme inhibiting agents were examined: cyanide ion, mercurous ion, sodium chloride, sodium fluoride, copper sulfate, lead acetate, alpha naphthol, and ethyl alcohol. The concentrations used were either hundredth or thousandth normal except for ethyl alcohol, which was employed in 30% concentration. Of these reagents, cyanide, mercurous ion, copper sulfate, alpha naphthol, and 30% alcohol had a significant effect on the reaction. Mercurous ion produced a marked accelerating effect (negative inhibition) on both stages of the reaction. Copper sulfate accelerated the initial reaction only. Cyanide inhibited both the initial and the mixing reaction, and a ten-fold increase in the cyanide concentration had little further effect on the amount of inhibition. Alpha naphthol inhibited the initial reaction very markedly, but had less effect on the mixing reaction; while 30% alcohol accelerated the initial reaction and almost completely inhibited the mixing reaction.

The alcohol inhibition was examined over a range of alcohol concentrations, from 5% to 40%, at two representative mixing times. The results are shown in Fig. 5. The acceleration of the initial reaction is constant for all concentrations from 5% to 35%, and at 40% marked inhibition of the initial reaction is evident. Increasing concentrations from 10% to 35% inhibit the rate of the mixing reaction, the rate being zero at 35% alcohol. At 40% alcohol there is a marked reversal of the reaction during mixing, which requires further investigation.

The alpha naphthol inhibition appears to be an antioxidant effect rather than true inhibition; for the initial reaction and a portion of the mixing reaction are completely inhibited, and the oxidation of pigment then proceeds again during a later phase of the mixing reaction. The alpha naphthol probably competes successfully with the pigment as

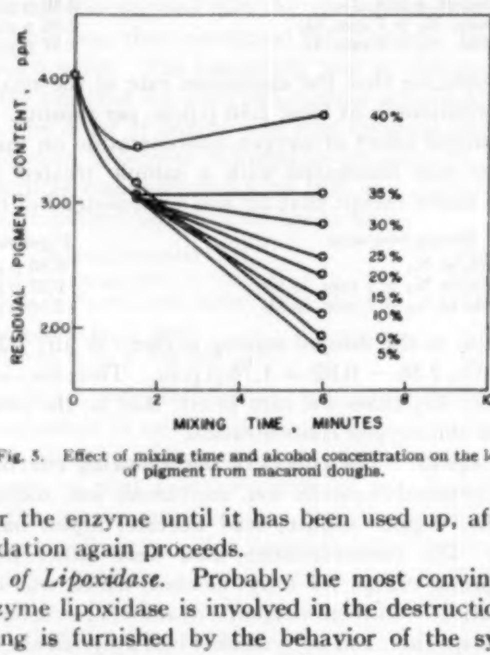

Fig. 5. Effect of mixing time and alcohol concentration on the loss of pigment from macaroni doughs.

substrate for the enzyme until it has been used up, after which the pigment oxidation again proceeds.

Addition of Lipoxidase. Probably the most convincing evidence that the enzyme lipoxidase is involved in the destruction of pigment during mixing is furnished by the behavior of the system on the inclusion of lipoxidase in the dough. The use of crude lipoxidase concentrates from soya bean meal for bleaching flour during the mixing of bread doughs has been common for over fifteen years. A sample of this concentrate bearing the trade name "Wytase" was obtained, and the amount recommended for bleaching bread flour doughs was added to several types of semolina before mixing. Two good varieties and one poor one were processed with this addition of enzyme. Added to the poor variety, the reaction rate was considerably increased but *the*

shape of the curve was the same as originally observed. The most striking effect was obtained with a sample of Mindum. The addition of "Wytase" produced a curve of the same type as that obtained normally with Golden Ball, a very poor variety. The control and "Wytase" curves are shown in Fig. 6 along with representative curves obtained for Mindum and Golden Ball. The data for the latter two samples are plotted, for ease of comparison, beginning at the same initial concentration; these values were actually 6.54 p.p.m. for Mindum, and 5.56 p.p.m. for Golden Ball. Thus it is possible, by the addition of a crude lipoxidase concentrate, to obtain the characteristics

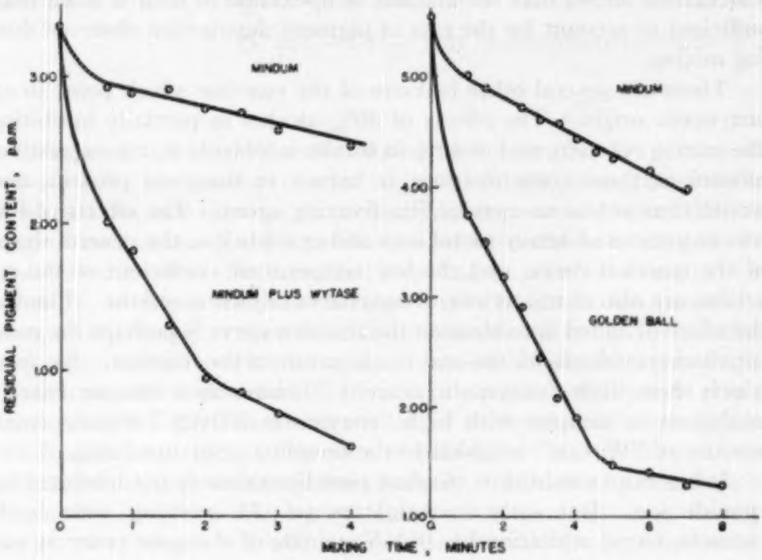

Fig. 6. Effect of added lipoxidase ("Wytase") on the loss of pigment for a good variety.

of a poor variety from a good variety and to make a poor variety considerably worse.

General Discussion

Destruction of the carotenoid pigments during mixing involves an oxidation reaction. This is evident from the pronounced effect of oxygen concentration on the mixing reaction, and from the complete inhibition of the initial reaction and part of the mixing reaction by alpha naphthol, an antioxidant. It seems extremely probable that the products of the oxidation are no longer carotenoid-like, as it has been shown (7) that macaroni contains the same pigments as semolina, but in smaller amounts. Thus the oxidation products appear to contribute nothing to the yellow color of the macaroni.

It is thought that the oxidation involves an enzymatic reaction. Oxidation of carotenoid pigments by atmospheric oxygen is normally a slow process (4). The action of peroxides such as exist in ether solutions of pigments is likewise slow, occurring over a period of weeks. In the reaction under investigation, the oxidation occurs rapidly in a few minutes, and the presence of an enzyme thus seems highly probable. The ability of soybean lipoxidase to oxidize carotenoid pigments during its peroxidation of unsaturated fats is well established, and the presence of this enzyme in wheat germ has been demonstrated by Sumner (11) and in commercial flour by Miller and Kummerow (9). Calculation shows that the amount of lipoxidase in flour is more than sufficient to account for the rate of pigment destruction observed during mixing.

There are several other features of the reaction which point to an enzymatic origin. The effects of 30% alcohol in partially inhibiting the mixing reaction, and of 40% in totally inhibiting it, are suggestive; alcohol in these concentrations is known to denature protein, and would thus act as an enzyme inactivating agent. The effects of low concentrations of heavy metal ions and cyanide ion, the general shape of the reaction curve, and the low temperature coefficient of the reaction, are also characteristic of enzyme catalysed reactions. Finally, the effect of added lipoxidase on the reaction curve is perhaps the most convincing evidence for the enzymatic nature of the reaction. Samples which show little "enzymatic activity" behave in a manner exactly analogous to samples with high "enzymatic activity" when a small amount of "Wytase" is added to the semolina prior to mixing.

It has been established (6) that pure lipoxidase is not inhibited by cyanide ion. But early investigators (cf. 3), working with crude extracts, found inhibition by .001 *N* cyanide of the same order as was observed in these experiments. Thus it appears probable that ***in vivo*** the enzyme functions in conjunction with an activating system that is sensitive to cyanide. This suggestion is further justified by the fact that a ten-fold increase in cyanide concentration had very little additional inhibiting effect. It is suggested, on the basis of evidence noted earlier, that the activating system is accelerated by mercurous ion and possibly by the cupric ion as well.

It is generally accepted that alpha naphthol does not act as a true inhibitor of the lipoxidase system, but rather as an antioxidant. This appears to be its function in the system under investigation.

If we accept the hypothesis that pigment destruction is brought about by a coupled reaction involving peroxidation of unsaturated fat by lipoxidase, it appears that there are two alternate paths for the reaction to follow: either the lipoxidase combines first with oxygen or

first with the unsaturated fat. The two reactions may be written as follows:

$$
\begin{array}{l}
\text{1. Lipoxidase} + O_2 \rightleftharpoons L{-}O_2 \\
\qquad\qquad\qquad\qquad\quad + \\
\text{Unsaturated fat} \rightleftharpoons L{-}O_2{-}UF \rightleftharpoons \text{intermediate peroxide} + L \\
\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad + \\
\qquad\qquad\qquad\qquad\qquad\qquad\quad \text{pigment} \\
\qquad\qquad\qquad\qquad\qquad\qquad\qquad \hookrightarrow \text{oxidized pigment} + \text{fat}
\end{array}
$$

$$
\begin{array}{l}
\text{2. Lipoxidase} + \text{unsaturated fat} \rightleftharpoons L{-}UF \\
\qquad\qquad\quad + \\
\qquad\qquad\quad O_2 \rightleftharpoons L{-}UF{-}O_2 \rightleftharpoons \text{intermediate peroxide} + L \\
\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad + \\
\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \text{etc.}
\end{array}
$$

The initial reaction appears to follow mechanism No. 1. It proceeds when mixing is carried out under nitrogen and also when the semolina is wetted but not mixed. Accordingly, it is postulated that the first step of mechanism No. 1, combination of lipoxidase and oxygen, has already occurred in the unwetted semolina in which the original cell structure of the wheat is still largely intact. It is also postulated that, at this stage, oxygen can diffuse into the cell but that lipoxidase and unsaturated fat are kept apart in immiscible phases; accordingly, the first step of mechanism No. 1 can take place but not the first step of mechanism No. 2. When water is added to the semolina it is rapidly imbibed and, according to the data of Baker, Parker, and Mize (2), the cells must rupture almost instantaneously. The subsequent steps of mechanism No. 1 can then occur. But the reaction soon ceases if either oxygen or mixing is withheld because the lipoxidase is not reactivated; for reactivation requires recombination of lipoxidase with substrate and oxygen which is thought to occur only as mixing exposes new surfaces of the dough to oxygen. Thus the initial reaction appears to be mainly a stoichemical one that is limited to the amount of lipoxidase-oxygen complex formed in the semolina before water is added.

As an alternative hypothesis, it may be postulated that the initial reaction involves prior formation of peroxides in the semolina, and that these react with the pigment when water is added. This explanation is not considered tenable; for it has been shown (10) that peroxides cannot oxidize the pigment directly, but that this is accomplished by some transient intermediate formed during peroxidation of fats by lipoxidase.

There is evidence that the mixing reaction can follow mechanism No. 2 rather than mechanism No. 1. When dough is mixed under nitrogen

and then under oxygen, pigment destruction is the same as if all the mixing had been done in oxygen. Thus, during the mixing reaction, mechanism No. 2 must be operative; for it is evident that, while no oxygen is being supplied, a complex is building up in the system during mixing which needs only oxygen for rapid completion of the reaction. This rapid step occurs when oxygen is supplied and given ready access to the dough by continued mixing. Since this reaction is so rapid it seems likely that mechanism No. 2 is the preferred reaction; if conditions are suitable, it will occur rather than No. 1. But if, as in semolina during storage, the first step of mechanism No. 2 is blocked, while the first step of No. 1 is not, then the initial reaction will still take place by mechanism No. 1.

The integrated hypotheses outlined in the preceding paragraphs account for the principal observations made during the course of the investigation. Since the fundamental postulate, that the reaction is catalysed by lipoxidase, was developed as a result of early experiments, it was partially verified by the final experiment: the effects of adding lipoxidase ("Wytase") were predicted and subsequently confirmed. Additional verification of this and other parts of the hypotheses must await the results of further study. Alternately, it is possible that alternate and simpler hypotheses, adequate to account for all the facts, may be developed.

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SOME CHARACTERISTICS OF THE STARCHES OF THREE SOUTH AMERICAN SEEDS USED FOR FOOD[1]

M. J. Wolf, M. M. MacMasters, and C. E. Rist[2]

ABSTRACT

Starch was prepared by laboratory processing from seeds of *Amaranthus leucosperma*, *Chenopodium quinoa*, and *C. paludicaule* which are closely related to tumbleweed (*A. graecizans* L.) and pigweed (*C. album* L.). These seeds were found to contain 62.8, 61.5, and 51.1% starch (d.b.), respectively. The starch in each case occurred as very small granules, approximately 1 to 3 μ in diameter. The starch from *A. leucosperma* colored reddish-brown with iodine-potassium iodide solution and sorbed only 2.5 mg. iodine per g., thus resembling the starches of waxy varieties of cereal grains. The starches from *C. quinoa* and *C. paludicaule* colored blue with iodine-potassium iodide solution and sorbed 45 and 50 mg. of iodine per g., respectively. The three starches gelatinized within the range 48°–72°C. Starch of *A. leucosperma* formed a "long" paste, *C. quinoa* starch a paste of the same "length" as that of corn starch, while *C. paludicaule* starch formed a thin, watery suspension. None of the pastes gelled on standing.

As part of a general study of the character of natural starches, the starches obtained from a number of native and foreign plant species have been examined. In the course of this work, seeds of *Amaranthus leucosperma*, *Chenopodium quinoa*, and *C. paludicaule* were processed for starch.

The characteristics of these starches may be of interest to cereal chemists since the seeds from which they were prepared are used in some areas of South America similarly to corn, wheat, and other cereals grown in the United States.

Seeds of *Amaranthus leucosperma* are popped, similarly to popcorn, for food use. This plant is related to the tumbleweed, *Amaranthus graecizans* L., which is native to the United States. *Chenopodium quinoa* seeds are used for making porridge and ground to flour for preparing bread and cakes. The seeds of *Chenopodium paludicaule* are ground into meal which is used for making porridge and also in mixture with wheat flour for baking. The latter two plants are related to the common Lamb's Quarters, *Chenopodium album* L. In the United States, seeds of some species of both *Amaranthus* and *Chenopodium*, including *C. album*, are collected by the Indians and made into meal for use in cakes or gruel (4, pp. 129, 140).

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It is well known that starches from different tissues, even of the same plant, may have different characteristics. Our common commercial starches come from the endosperm of cereal seeds, and from modified roots and stems of several other plants. The three starches reported here may be of particular interest because they are stored in the perisperm of the seed. Little is known about starch from this type of tissue.

Results and Discussion

Like our common cereal grains, these South American seeds contain starch as a major constituent. The amount of starch present is of the same order as that in wheat, but is appreciably less than in corn (Table I). Unlike the cereal grains, however, in which the starch is stored in the cells of the endosperm, the storage tissue in these seeds is the perisperm. In mature cereal grains the perisperm as a distinct tissue is completely lacking or is present only in negligible amounts and is of no importance as a starch storage tissue.

TABLE I

STARCH AND PROTEIN CONTENTS OF SEEDS AND SOME PROPERTIES OF THE STARCHES

Seed			Starch[1]			
Botanical name	Starch content[2]	Protein content $N \times 6.25$	Nitrogen content[3]	Granule diameter[4]	Color with $I_2 \cdot KI$ solution	I-sorptive capacity[5]
	% (*d.b.*)[6]	% (*d.b.*)[6]	% (*d.b.*)[7]	μ		*mg./g.*
Amaranthus leucosperma	62.8	16.1	0.13	1–3.5	Reddish-brown	2.5
Chenopodium quinoa	61.5	15.3	.17	1.5–3	Blue	45
Chenopodium paludicaule	51.1	16.9	.12	1–3	Blue	50
Corn	70.9	9.9	.04	1–23	Blue	54
Wheat	64.4	14.7[8]	.04	2–40	Blue	50

[1] Starch was separated from the seeds by the second method described by MacMasters and Hilbert (3); this is a laboratory wet-milling procedure using only distilled water.
[2] Determined polarimetrically in a calcium chloride extract of the dry, ground seed (2).
[3] Method of (1) with Winkler's modification (6).
[4] Determined microscopically with the aid of a filar-micrometer eyepiece.
[5] Method described in (7). The values given are calculated on a protein-free basis.
[6] Moisture content was determined by drying the ground seeds for 1½ hours at 130 C. in a forced-draft oven.
[7] Moisture determined as in (7).
[8] $N \times 5.7$.

The protein content of the seeds is similar to that of wheat but is considerably higher than that of corn (Table I). The high residual nitrogen content of the *Amaranthus* and the two *Chenopodium* starches indicates difficulty in separating protein from the small starch granules by the method used.

With respect both to color reaction with iodine-potassium iodide and iodine-sorptive capacity, the two *Chenopodium* starches are like the

TABLE II

COMPARISON OF SOME PHYSICAL CHARACTERISTICS OF AMARANTHUS AND CHENOPODIUM STARCHES WITH THOSE OF CORN AND WHEAT STARCHES

Source of starch	Gelatinization range,[1] °C.	Relative paste length[2,3]	Gel formation[2,4]
Amaranthus leucosperma	55–72	Considerably longer than corn starch	None
Chenopodium quinoa	48–62	Short; about same length as corn starch	None
Chenopodium paludicaule	56–71	Shorter than corn starch; watery	None
Corn	52–72[5]	Short	Firm
Wheat	51–64[6]	Short	Soft

[1] Temperatures at which gelatinization starts and at which it is complete, respectively. Determined by slowly heating the starch suspension in a water bath and withdrawing samples for microscopic observation at intervals. Because the small size of *Amaranthus* and *Chenopodium* starch granules precluded the use of conventional methods, gelatinization range was determined by noting changes in size, form, and transparency of the granules. These data for corn and wheat starches were determined by loss of birefringence and by staining with benzopurpurin.

[2] All starches were defatted.

[3] Estimated by heating 5% starch suspension over a boiling water bath until thickened, cooling to about 30°C., drawing up the paste with a glass rod, and noting the length of the strand formed.

[4] Noted after storing the pastes at about 8°C. for 24 hours.

[5] Average values for many commercial samples.

[6] Average values for many laboratory-processed samples.

common starches of commerce, while the *Amaranthus* starch is similar to the starches of the waxy varieties of cereal grains (Table I).

The starches gelatinized over approximately the same range as ordinary corn and wheat starches (Table II). *Quinoa* starch gelatinized over a somewhat lower temperature range than the other two starches. The waxy character of the *Amaranthus* starch is emphasized by the long paste which it forms in comparison to non-waxy corn starch. The failure of the waxy *Amaranthus* starch to gel may be anticipated; however, the two non-waxy *Chenopodium* starches also yielded no gel on standing. Small granule starches from other plants, such as dasheen and rice, have been observed also to form either weak gels or none at all.

More than a dozen plants are mentioned in Meyer's (5) summary of the sources of starches which are colored red in the presence of iodine solution. Waxy corn, waxy *Coix*, and waxy barley starches have been added to the list since that time. As far as we know, however, *Amaranthus leucosperma* is the first plant of commercial importance, other than the cereal grains, which has been found to contain this type of starch.

Acknowledgment

The samples of seeds studied were made available to us through the courtesy of Dr. Hugh Cutler, Chicago Museum of Natural History, who collected them in or near Cochabamba, Bolivia.

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KERNEL HARDNESS IN CORN. I. A MACHINE FOR THE RAPID DETERMINATION OF KERNEL HARDNESS[1]

Edmond H. Bennett[2]

ABSTRACT

An electrically operated machine has been devised for the rapid determination of hardness of grain. A mechanical feeder delivers grain at a uniform rate between an inner driven wheel rotated at 33 r.p.m., and an outer wheel which rotates only when grain is being crushed, since it is propelled by the pinning action of the crushing grain.

Indexes to hardness are obtained by a hydraulic piston-regulated, recorder-unit which is driven by the outer crusher wheel when it rotates. The hydraulic pressure is generated in an hydraulic cylinder, the plunger of which is actuated by the torque transmitted to the crusher frame by the crusher wheels. Either the number on the recorder or the hydraulic pressure, as registered on a pressure gauge, may be used as an index of hardness.

The coefficient of variability ranged between one and three per cent for tests on samples of corn.

For some years livestock feeders and many agricultural investigators have been interested in the determination of corn hardness to ascertain the relationship between hardness and feeding value. Moreover, the rapid determination of seed hardness should be of value, particularly to agronomists and millers, since hardness is related to certain physical and chemical properties.

Various methods have been used to arrive at indexes of corn hardness. Robison (4) compared the per cent of starchy kernels in hybrids and open-pollinated varieties of corn. The kernels were examined over a glass-topped box containing an electric light. If the opaque

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area extended the full length of the kernel it was termed starchy. Culbertson, Shearer, Hammond, and Robinson (2) measured the hardness of kernels on edge between the jaws of a machine applying pressure until the kernel was crushed. Veach (5) built an hydraulic seed hardness tester and used it on various kinds of seeds, including clover, Lespedeza, sweet clover, and corn. Bennett (1) used the Veach machine to study the influence of such variables as shape, size, moisture content, and storage time on the crushing strength of corn. All of the tests mentioned above were made on a number of individual

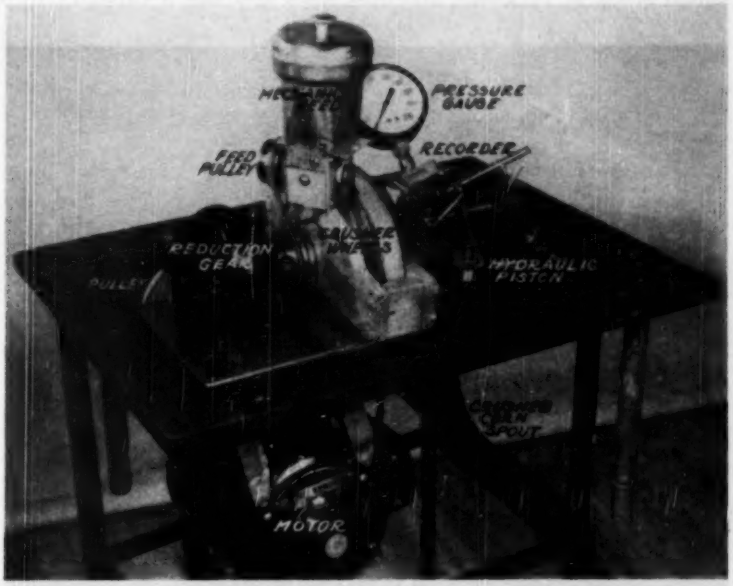

FIG. 1. Left rear view of crusher. Details of mounting on steel and wood platform are shown. The legs on the platform make possible the removal of the crusher from its stand to a table or bench if desired. The stand has rollers which make it easily portable. The flexible crushed corn spout, shown at lower right, carries the corn away. The rear end of the frame is raised a variable distance in crushing, while the front lowers and forces a piston downward.

kernels. Bennett (1) also determined corn hardness by a method developed by Cutler and Brinson (3) for testing wheat. This method is based on the fact that a ground sample of hard seeds yields a higher per cent of large sized particles than softer seeds.

The early attempts to determine corn hardness by crushing a number of kernels individually and using the average crushing pressure proved slow and arduous. The extreme variability of hardness made it necessary to test large numbers of kernels to obtain means with low errors. Three machines were built successively in the attempt to perfect a simple, dependable method of obtaining quick, accurate, and

valid tests by utilizing *aggregate* samples. This paper describes the construction and operation of the third machine, the one currently in use.

Materials and Methods

Fig. 1 is a photograph of the machine, left rear view, and Fig. 2, a photograph of a plan view. The principle of operation is illustrated by the schematic drawing, Figs. 3 and 5. All the metal parts of the machine are polished and cadmium plated to prevent corrosion.

Automatic Feeder. A sample of seed is placed in the hopper which is constructed of 16 guage stainless steel and has a capacity of ap-

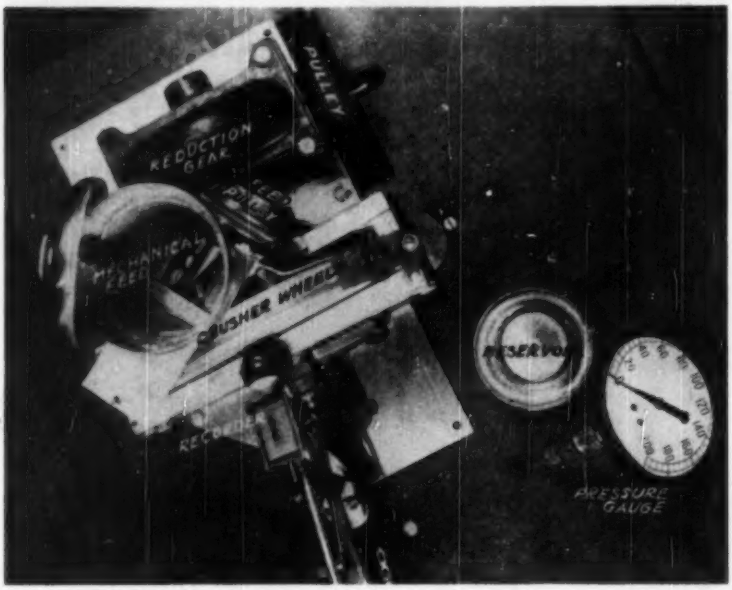

FIG. 2. Plan view of crusher. Through the open hopper may be seen the details of the mechanical feed. Position of recorder on the outer crusher wheel is also shown. Three kernels are shown in one of the roller groves.

proximately 300 gms. of corn. The automatic feeder then delivers the seeds in a uniform stream to the crusher wheels. Because the seeds must be delivered to the crusher at a uniform rate, the automatic feeder is a very important part of the machine. Fig. 2 shows the diagonally grooved roller located at the bottom of the hopper. This roller feeds the seeds to the crusher. It is driven from the drive shaft of the crusher wheel by means of sheaves and a coiled spring belt. Four roller speeds are possible by the use of two steps on each sheave. Rate of feed may be further regulated or shut off entirely by means of a steel plate which slides into the bottom of the hopper.

Crushing Mechanism. The seeds are crushed between the outer surface of the inner wheel and the inner surface of the outer wheel. The position of the wheels (Fig. 4) is made possible by supporting each wheel independently in a rigid steel frame. The inner wheel (5 in. d. $\times \frac{9}{16}$ in. w.) is driven by, and is integral with a shaft (Fig. 4, No. 2). This shaft turns in a bronze bearing and is attached by a rigid coupling

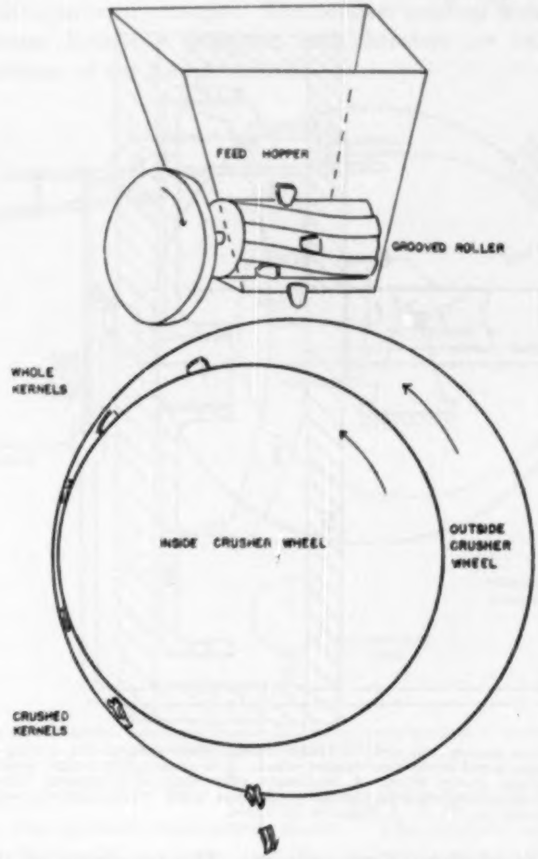

FIG. 3. Schematic drawing of crusher, showing principle of operation.

to the drive shaft of the speed-reduction gear. The outer crusher wheel (7 in. d. $\times \frac{9}{16}$ in. w.) is supported by an eccentric shaft (Fig. 4, No. 4) on the crusher frame, opposite the drive shaft of the inner wheel. The shaft surface is eccentric in relation to its support in the crusher frame so that the distance between the crushing surfaces can be regulated from 0 to ¼ in. by turning the shaft in the frame. The

desired clearance is maintained by tightening the shaft retaining nut. The outer wheel turns freely on the outer bronze bearing surface of the shaft. The crusher frame has holes drilled to the bronze bearings, fitted with Alemite grease fittings for lubrication. The frame (Fig. 4, No. 5) is free to rock about its two concentric supports (Fig. 4, No. 6). These bearing supports are contained in pedestals welded to the base plate of the apparatus.

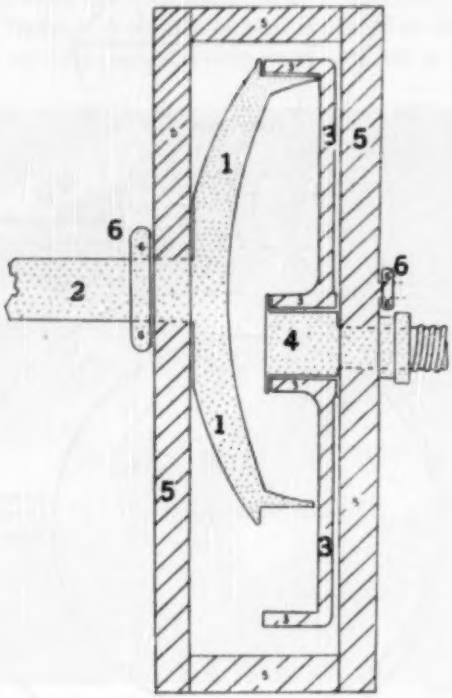

FIG. 4. Cross section, top view. 1. Inner crusher wheel integral with driving shaft No. 2. 2. Shaft which supports and drives inner crusher wheel. It is connected by a rigid coupling to the reduction gear. 3. Outer crusher wheel. 4. Stationary, offset shaft which supports outer crusher wheel. Turning this shaft adjusts crushing clearance. 5. Steel frame which supports crusher wheels, and rocks in frame supports, No. 6. 6. Supports for frame.

Principle of Force Transmission. The resistance of the seeds to crushing produces separating forces acting upon the two crusher wheels. These forces will occur in the "crushing zone" (Fig. 5) and their resultant will be the equal and opposite forces "A" acting on the two wheels.

These forces are transmitted through the two crushing wheels to their respective bearings in the frame. The forces acting on the frame are labeled "B" in Fig. 5, and constitute a couple or torque equal to the

product BL which tends to rotate the frame (Fig. 4, No. 5) in its supports (Fig. 4, No. 6). "L" is the distance between the two lines of force "B" (Fig. 5).

The frame is restricted from rotating by means of hydraulic pressure on an hydraulic piston (Fig. 5). The hydraulic pressure (which is proportional to the frame torque and an index to seed hardness) is transmitted to a pressure gauge, and also to a mechanical integrating device which actuates a counter. The counter reading is proportional to the average hydraulic pressure, and therefore an index of the average hardness of the sample tested.

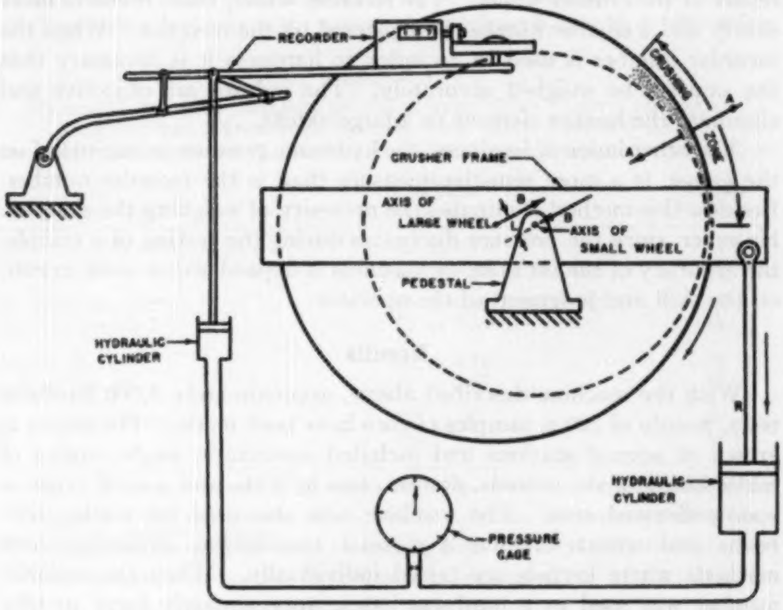

FIG. 5. Schematic drawing of crusher illustrating forces involved in crushing corn and in measuring the forces.

Recording of Forces. The recorder is composed of the integrating device and the counter mentioned above. The integrating device is actuated by hydraulic pressure, and the counter is turned by a rubber wheel which rolls on the side of the outer crusher wheel when seeds are being crushed. A rod (R) connects the crusher frame to an hydraulic piston. This piston produces an hydraulic pressure proportional to the torque developed, and is registered on a pressure gauge in units of 0.15 lbs. per sq. in. The pressure fluctuates during the testing of a sample. This is especially true with corn. The effect of these fluctuations on the indicator hand of the gauge is dampened by the metering effect of a

constriction produced by a small hole drilled through a block installed in the line of the gauge. Copper tubing transmits the hydraulic pressure also to a second cylinder where the force on a piston regulates the position of the recording device (modified r.p.m. counter) working on the outer side surface of the outer crusher wheel (Figs. 1, 2, and 5). The greater force required to crush hard seeds causes the recorder to operate near the periphery of the outer crusher wheel. The recorder wheel, therefore, revolves rapidly; and a relatively large number is registered on the recorder for a definite weight of seeds. Soft seeds, on the other hand, need less force and the recorder operates nearer the center of the crusher wheel. The recorder wheel, then, revolves more slowly and a smaller number is registered on the recorder. When the recorder number is used as an index to hardness it is necessary that the samples be weighed accurately. The indexes are objective and eliminate the human element to a large extent.

The other index of hardness, the hydraulic pressure as registered on the gauge, is a more sensitive measure than is the recorder number. Besides, this method eliminates the necessity of weighing the samples. However, since the pressure fluctuates during the testing of a sample, the accuracy of the estimate of hardness is dependent, to some extent, on the skill and judgment of the operator.

Results

With the machine described above, approximately 3,000 hardness tests, mainly of 200 g. samples of corn have been made. The corn was grown at several stations and included systematic single crosses of midseason inbreds, inbreds, double cross hybrids, and special types of open-pollinated corn. The machine was also used for testing soybeans and wheat. It has a distinct time-saving advantage over methods where kernels are tested individually. When the recorder number was used as a hardness index, approximately forty to fifty samples per hour were weighed and tested by an experienced operator. When the hydraulic pressure was used directly as a hardness index, testing was more than twice as fast.

Validity. The results agreed with the physical appearance believed to be associated with hardness in corn. Illinois "high protein" corn has small kernels which appear very dense and have little or no floury endosperm. When tested it proved considerably harder than Illinois "low protein" which has larger kernels with the endosperms almost entirely floury (Table 1). Inbred 38–11 which was considered to contribute hardness to hybrids, proved much harder than inbred Indiana 66, which was considered to contribute softness to hybrids. A hard and a soft hybrid corn was tested for hardness on

both the above described machine and the Veach (5) machine, mentioned earlier, to compare their results with the physical appearance of the corn. The two hybrids were bred especially for testing purposes. One (38–11 × 5677) (307 × Kys), was grown from inbreds selected for their ability to contribute hardness to the hybrids in which they were used. The inbreds which made up (WF9 × CC1) (Ind. 66 × Hy) were believed to contribute relative softness to hybrids. The physical appearance of the two hybrids is illustrated by Fig. 6. When they were tested for hardness with the machine described in this paper, the softer appearing hybrid had an index of 56, on the recorder, and the harder hybrid had an index of 72. However, when individual kernels of these same hybrids were tested on the Veach machine, the softer

TABLE I

Hardness Tests on Samples of Grain from Corn Grown at Illinois Agricultural Experiment Station, 1944, Each Test 200 Grams

Corn	Mean Recorder Score	Pressure Readings
(38–11 × 5677) (307 × Kys)	72[1] ± 0.73[2]	92[3]
(WF9 × CC1) (Ind. 66 × Hy)	56 ± 0.41	58
Ill. "high protein"	60 ± 0.55	65
Ill. "low protein"	52 ± 0.34[2]	48
Inbred 38–11	62 ± 0.70	70
Inbred Ind. 66	51 ± 0.80	44
hh (defective endo.)	52[4]	40
fl_2	48[4]	35

[1] The recorder score represents, in effect, the product of an average crushing pressure and duration of test.

[2] Standard error of mean recorder score of three to seven tests, the number depending on the amount of corn available for testing.

[3] While testing, the operator estimates the average pressure from the gauge, expressed in units of 0.15 p.s.i.

[4] Sufficient corn for only two tests was available from these samples.

appearing hybrid (WF9 × CC1) (Ind. 66 × Hy) tested considerably harder than the other. An examination showed that a smaller surface area of the rounded kernels of the harder hybrid (38–11 × 5677) (307 × Kys) touched the flat, parallel jaws of the machine than was the case with the softer hybrid. It is thought that the smaller contact area caused a localization of stress which allowed the more rounded kernels to be more easily crushed even though they were harder than the flat ones of (WF9 × CC1) (Ind. 66 × Hy). Results indicate that the rounded crushing surfaces of the machine described in this paper tend to minimize the effect of kernel shape on crushing resistance.

Variability of Individual Kernel Tests Compared With Aggregate Tests. The most striking observation made while testing hardness by

crushing individual corn kernels with several individual kernel machines was the extreme difference in their hardness. When such tests were run using the Veach machine on the above mentioned hybrids, 120 kernels of (WF9 × CC1) (Ind. 66 × Hy), with a mean crushing resistance of 164.5 lbs. had a standard error for individual tests of ±97.0 lbs. One hundred twenty kernels of (38–11 × 5677) (307 × Kys), with a mean crushing resistance of 109.0 lbs. had a standard error for individual tests of ±86.0 lbs. Similar tests were run on inbred 38–11 to determine whether inbreds varied as much as hybrids. One hundred kernels had a mean crushing resistance of 112

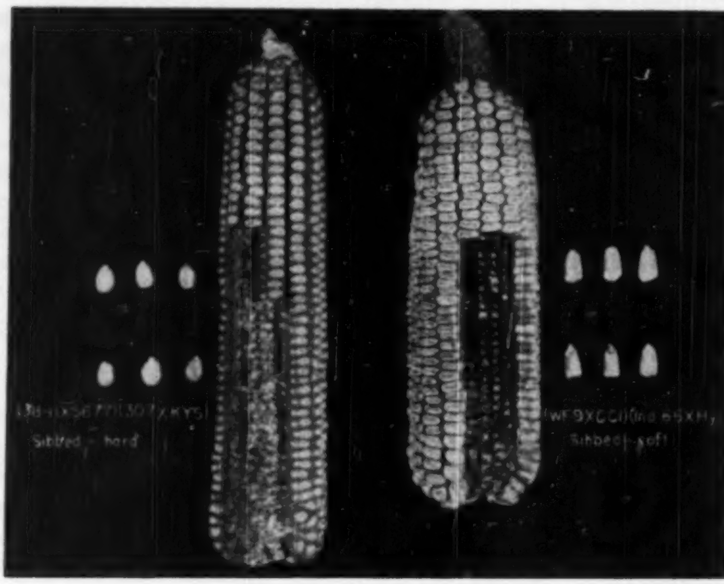

FIG. 6. Two hybrids produced from inbreds selected for their hardness contributing qualities.

lbs. with a standard error of 56.0 lbs. It was found in testing Illinois Station Yellow Dent, very closely graded for size through both round and long holed screens, that it was necessary to test 273 kernels to obtain means that were significant at the 5% level, within the limits, ±10.0 lbs.

Using the recorder score of the machine under consideration, hybrid (38–11 × 5677) (307 × Kys) with a mean hardness index of 72.0, had a standard error of ±1.29 for individual tests. Hybrid (WF9 × CC1) (Ind. 66 × Hy), with a mean hardness index of 56.0, had a standard error for individual tests of ±0.71. Although the units of measurement differ in the two types of tests, it is evident that the

machine described here, has materially reduced the error of hardness tests.

Other Seeds. When the crusher was used for testing soybeans no change was found necessary in the crusher clearance or the rate of feed. Soybeans proved softer than corn, and there was less fluctuation in crushing pressure. However, tests showed considerable difference between the varieties tested. Tests made on wheat without changing the settings from those used with corn were not satisfactory. Some of the wheat passed through without being crushed and the feeder delivered the wheat to the crusher too fast. With appropriate adjustments, however, it is possible that wheat also could be tested accurately.

Acknowledgment

The author expresses his thanks to G. H. Dungan, Professor of Crop Production at the University of Illinois, for advice concerning Agronomic problems. He also thanks Professors S. Konzo, R. C. Juvinall, and C. D. Greffe of the Department of Mechanical Engineering, for helpful advice concerning engineering terminology and description.

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KERNEL HARDNESS IN CORN. II. A MICROSCOPIC EXAMINATION OF HARD AND SOFT TYPES OF DENT CORN[1]

EDMOND H. BENNETT[2]

ABSTRACT

A close correlation was found between observed structure of the corn kernels and hardness as measured with the machine described in the foregoing article. Mature kernels of hard types of dent corn have smaller starch granules and more dense appearing protein matrix than softer types of dent corn. The amount of floury endosperm was found to be greater for the softer corns.

In wet milling, the aims are to remove all the starch from its matrix, and to remove all other cell contents from the starch. The more incomplete their separation, the poorer is the quality of the starch, or the lower is the per cent of starch return from corn. In the production of corn flakes, large grits are sought that are free from checks. Such grits, when properly softened, can be rolled out into large uniform flakes. Corns vary in the extent to which they satisfy the above demands, undoubtedly due to structural differences. Since starch and its proteinaceous matrix form most of the endosperm, their relationship is important in a study of its structure.

Cox, MacMasters, and Hilbert (3) observed that the smallest starch granules were found in the cells immediately beneath the aleurone layer where they are imbedded in a heavy protein matrix. They also noted that during processing most of the granules from this location contributed to tailings and relatively few to the prime quality section of starch.

It is the purpose of the present study to compare the structure of kernels which contrast widely in hardness, and point out the possible significance to the processing of corn.

Materials and Methods

Samples of mature kernels from three groups of corn were tested for hardness employing the machine described by Bennett (1). The hardest and softest from each group, as well as four other corns were selected for microscopic examination of structural characteristics.

[1] Manuscript received August 12, 1949. From the Department of Agronomy, University of Illinois, Urbana, Illinois.
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These included: a rice type popcorn, a Peruvian flour corn, "Cuzco";[3] two double cross hybrids, one bred for its hard and the other for its soft characteristics; one hard and one soft single cross hybrid; one hard and one soft inbred; Illinois "high protein" corn with a protein content of approximately 20%, relatively hard, and Illinois "low protein" corn, relatively soft, with a protein content of approximately 5 to 6%.

Sectioning. The mature corn kernels were soaked 28 hrs. in distilled water at 36°C. and sectioned with a sliding microtome using a carbon

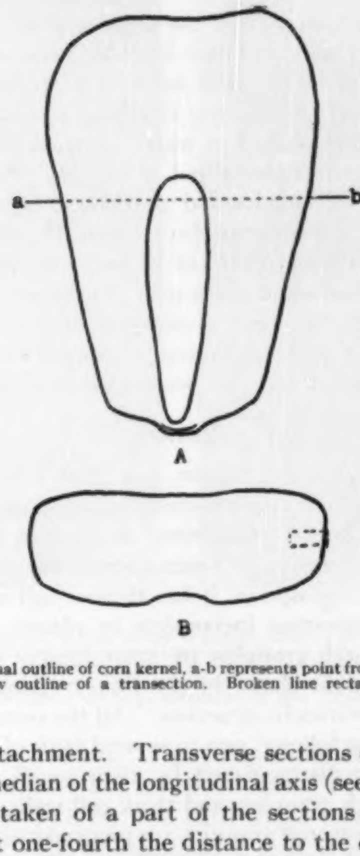


FIG. 1. A. Longitudinal outline of corn kernel, a-b represents point from which transverse sections were taken. B. Face view outline of a transection. Broken line rectangle indicates area photographed.

dioxide freezing attachment. Transverse sections of the corn kernels were made at the median of the longitudinal axis (see Fig. 1A). Photomicrographs were taken of a part of the sections from the aleurone layer inward about one-fourth the distance to the center (Fig. 1B) of the sections. The sections were approximately 20 μ in thickness, with the exception of popcorn which was about 12 μ, and Cuzco, which was about 80 μ. The sections were stained with safranin and

[3] Popcorn and Cuzco were not tested for hardness but were examined microscopically because they represent, respectively, the extreme flinty type and the extreme floury type of endosperms.

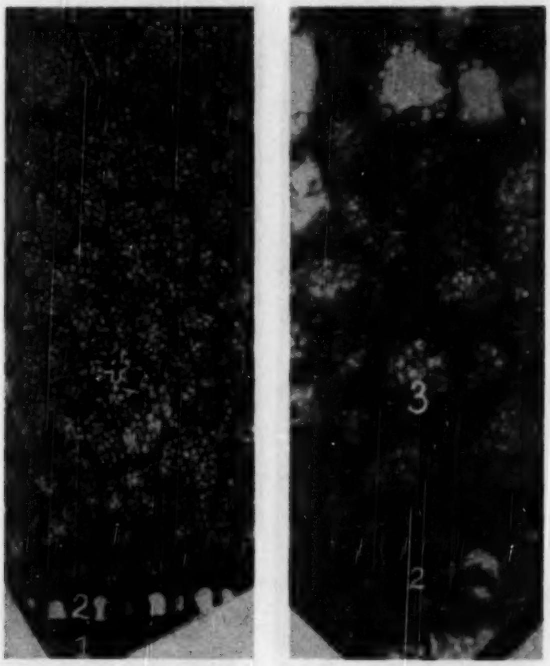
haematoxylin and very lightly with iodine (ten p.p.m.). The staining procedure was regulated so that the assembled colors were: pericarp—bright red; aleurone layer—dark with some cells clear where the contents had dropped out; cell walls—dark violet to brown; protein matrix—light yellowish brown; embryo—violet; starch granules—very light blue; nuclei—dark violet. Some sections were stained as above but with iodine omitted from the procedure and the coloration of the nuclei was the same as those with the iodine included. The sections were mounted temporarily in water and photomicrographs made.

Starch granules from each of the corns studied, with the exception of the two single crosses and two inbreds, were examined under the microscope and measured with an ocular micrometer. The final measurement was calculated after checking with a stage micrometer. The kernels were first soaked in water, then sectioned with a sliding microtome at the location indicated in Fig. 1B. Strips were cut from three areas of the sections located as follows: area I was located immediately beneath the aleurone layer; area II, midway between the aleurone layer and the center of the kernel; and area III, in the center of the section. These small strips were placed on a microscope slide in a drop of water and the starch granules teased out. A drop of dilute iodine solution with a concentration of approximately ten parts per million was then added and the measurements made.

Results

A cross section of a dry mature dent corn kernel shows the endosperm with two distinct regions, the horny endosperm and the floury endosperm. The floury endosperm is opaque, friable and easily crushed between the fingers. Upon microscopic examination, in contrast to the horny endosperm, it has thinner cell walls and less dense protein matrix, appearing incomplete in places, around the starch granules. The starch granules are more loosely arranged and more uniformly spherical in shape in the floury endosperm. The horny endosperm, itself, varies in structure. All the corns examined showed a general pattern as follows: one to several rows of cells just inside the aleurone layer are distinguished by their small size, dense protein matrix, small starch granules, and thick cell walls. Centripetally the cells, as well as the starch granules are progressively larger in size, and more variable in size and shape. The starch granules are progressively more crowded farther from the aleurone layer toward the inner limits of the horny endosperm. The closely packed granules become distorted in shape in contrast to the roughly spherical shape of the less crowded granules nearer the aleurone layer.

Popcorn and Cuzco. The present study confirmed structural differences found by Cox *et al.* (3) between popcorn and Cuzco. Starch granules were smaller and the protein matrix more dense in the horny endosperm of popcorn than for corresponding areas in the horny endosperm of Cuzco. These differences are illustrated by Fig. 2. The nuclei in the cells of popcorn endosperm are considerably larger.



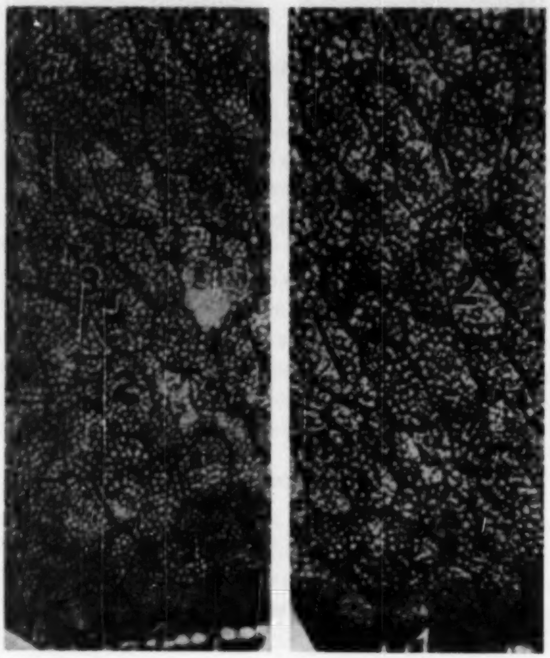
A B

FIG. 2. A. Photomicrograph of cross section of popcorn showing cellular relationship. 1. Pericarp; 2. Aleurone layer; 3. Cell of horny endosperm; 4. Nucleus. × 100. Thickness 12 μ. B. Cross section of Cuzco corn kernel. Pericarp (1) is shown at bottom. Aleurone layer (2) is two cells thick in places. Cells (3) are larger than those of popcorn. Starch granules are larger. Upper left corner of photomicrograph includes the edge of the floury endosperm. × 100. Thickness approx. 80 μ.

Dent Hybrids and Inbreds. Fig. 3 illustrates the structural differences found between hard and soft dent corn. The hard double cross (38–11 × 5677) (307 × Kys), the hard single cross (L317 × 38–11), and the hard inbred (Ill.90) contained a greater proportion of horny endosperm than did the softer corns (WF9 × CC1) (Ind. 66 × Hy), (K155 × WF9) and CC1. For corresponding areas within the horny endosperms of the harder corns the protein matrix appeared more dense, the starch granules smaller and the nuclei more prominent than

in the softer corns. The nuclei of the hard inbred, Ill.90, were not as large as those of the two hard hybrids.

Illinois "high protein" and Illinois "low protein." The contrast in macroscopic appearance between sections of Illinois "high protein" kernels and sections of Illinois "low protein" kernels was almost as great as between sections of popcorn and Cuzco kernels. The kernels of "high protein" were the smaller and harder of the two. They con-



A B

FIG. 3. A. Photomicrograph of cross section of hard hybrid (307 × Kys) (38–11 × 5677) corn kernel. 1. Aleurone layer; 2. Endosperm cells; 3. Nuclei. × 100. Thickness approx. 20 μ. B. Cross section of soft hybrid (WF9 × CC1) (Ind. 66 × Hy). Aleurone cells (1), endosperm cells (2), and starch granules are larger than those of the harder hybrid. Nuclei are less conspicuous. × 100. Thickness approx. 20 μ.

tained a small amount of floury endosperm at the crown, but very little or none back of the scutellum. The floury endosperm of "low protein" kernels extended from the distal to the chalazal end and in some kernels the horny endosperm occurred only as a thin shell around the floury endosperm. Microscopic examination showed that "low protein" kernels possessed larger starch granules in areas I and II (Fig. 4). However, the starch granules from the center (area III) of "low protein" kernels were smaller than for area II, and the starch granules

from area III of "high protein" kernels were larger than those found anywhere else in the same or different kinds of corn that were examined

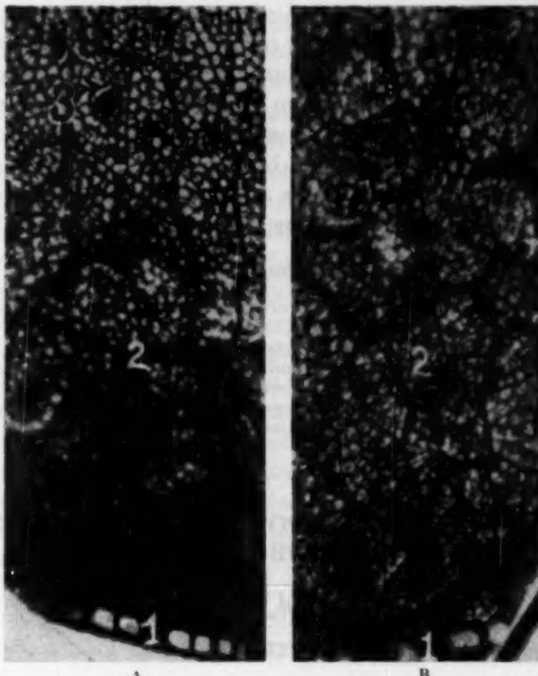


FIG. 4. A. Photomicrograph of cross section of Illinois High Protein kernel. 1. Aleurone cells; 2. Endosperm cells; 3. Prominent nuclei. × 100. Thickness approx. 18 μ. B. Illinois low protein kernel. 1. Aleurone cells. 2. Endosperm cells. Starch granules are larger in the area shown above. Nuclei are less conspicuous. × 100. Thickness approx. 25 μ.

in the present work. The protein matrix was more dense in the "high protein" corn, and the nuclei were especially large and conspicuous.

Discussion

The results of the present work with dent corn indicate that hardness is related to certain structural characteristics. The harder corns examined had smaller starch granules in a denser appearing matrix than the softer corns. The work of Cox *et al.* (3) indicated that the smaller starch granules in a massive protein matrix were contributed to tailings. In softer corn with its larger starch granules the separation of starch should be more complete resulting in a higher per cent of starch return and a greater percentage of high quality starch. This expectation needs to be verified by further tests, however.

Although considerable effort is expended on methods of processing corn to produce better finished products, present methods of quantity buying discourage attempts to determine the characteristics that are desirable in the raw product. Since wet millers normally produce starch from low, or sample grade corn, it seems improbable that the present research will directly influence their choice of raw material. The main conclusion to be drawn from the work presented here is that the differences found by Cox *et al.* (3) between popcorn and flour corn also occur, in a less degree, between hard and soft varieties of dent corn. This may be useful as a partial explanation of differences observed by wet millers in steeping different lots of corn.

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AMINO ACID COMPOSITIONS OF WHEAT AND CAROB GLUTENS [1]

A. C. Rice [2] and P. E. Ramstad [3]

ABSTRACT

A material resembling wheat gluten was prepared from the germ flour of the carob seed, *Ceratonia siliqua*. Hydrolysates of this preparation and wheat gluten were analyzed for their contents of 17 amino acids using microbiological methods. Reasonably good agreement was found between results for wheat gluten by this procedure and those previously reported in the literature. Carob gluten differed from wheat gluten in containing much more arginine, aspartic acid, and lysine; somewhat more glycine and histidine; somewhat less cystine, glutamic acid, and phenylalanine; and much less proline. It is apparent that similar physical properties may be shared by proteins of widely varying amino acid composition.

The unique physical properties of wheat gluten are in large part responsible for the behavior of wheat flour in baked goods. Flours of other cereal grains do not possess these properties to the same degree and cannot be satisfactorily substituted for wheat flour. Variations exist in the properties of glutens depending on the type of wheat and the grade of the flour.

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Received for publication October 3, 1949.
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Many workers have studied the chemistry of the wheat proteins in an effort better to explain their physical properties and account for variations in these properties. The literature in this field has been reviewed by Bailey (2). Differences have been observed in chemical composition of proteins from various flours, but correlation of these with physical behavior proved difficult.

Bienenstock *et al.* (3) have reported that proteinaceous materials possessing the physical properties of gluten may be prepared from the germ tissues of seeds of certain members of the legume family. One of these is the carob bean, *Ceratonia siliqua*, indigenous to the Mediterranean area. The carob bean pod is used in livestock feeds; the seed endosperm when ground is the product known as locust bean gum, and the seed embryo is used in Europe both in feeds and human food. The embryo contains no starch but has a very high protein content, over 50%. When the embryo is ground to a flour, it may be made into a dough with water. This dough, if carefully washed to remove nonproteinaceous materials, will yield a "gluten" which closely resembles wheat gluten in appearance, extensibility, and elasticity.

It seemed of interest to prepare some of this carob gluten and compare its amino acid composition with that of wheat gluten to ascertain whether or not two materials of such outward similarity were also similar in composition.

Materials and Methods

Wheat flour used as a source of gluten was a commercial strong baker's patent.

Carob germ flour was prepared from carob seeds. Seed coats were removed by carbonizing in concentrated sulfuric acid, washing in water, and drying. Germ was separated from endosperm by a grinding and sifting procedure, taking advantage of the fact that the endosperm was very hard and tough, while the germ was much more friable. The germ was finally ground and bolted through a 10 xx silk bolting cloth. The germ flour recovered comprised 22% of the weight of the seeds.

Both the wheat and carob flours were extracted for 16 hrs. with petroleum ether (b.p. 30–60°C.) in a Soxhlet extractor.

Gluten was prepared from the wheat flour by the A.A.C.C. (1) method. A similar procedure was used for the carob germ flour except that 140% absorption was required in making the dough.

Following the determination of moisture content and nitrogen (Kjeldahl) according to A.A.C.C. methods (1), hydrolysates of the carob and wheat glutens were prepared. Acid and alkaline hydrolysates were made, using 20 ml. of 10% hydrochloric acid per gm. of sample for the former and 20 ml. of sodium hydroxide per gm. of sample for

the latter. The samples varied in weight from 1–1.5 gms.; the dry weight, however, was from 0.3–0.5 gms. Hydrolysis was carried out in sealed ampules at 15 lbs. pressure for 16 hours. After neutralization, the hydrolysates were filtered or centrifuged, made to volume with distilled water (acid hydrolysate, 100 ml.; alkaline hydrolysate, 200 ml.), and stored under toluene at 2°C.

The hydrolysates were analyzed microbiologically for those amino acids indicated as being present in wheat gluten by Block and Bolling (4). The alkaline hydrolysates were employed for the assays of tyrosine and tryptophan, while the acid hydrolysates were used for the remainder of the assays. The media of Steele *et al.* (10) and Stokes *et al.* (11) were used with slight modifications. A stock vitamin mixture was used, differing slightly from that of both authors, which was as follows: thiamine·HCl—250γ, pyridoxamine·HCl—500γ, pyridoxal·HCl—500γ, Ca-dl-pantothenate—250γ, riboflavin—250γ, niacin—500γ per 250 ml. of medium.

Organisms used included *Streptococcus faecalis*, *Leuconostoc mesenteroides* P-60, and *Leuconostoc citrovorum*, 8081.[4] These were transferred to a liver-tryptone broth (6) 16–24 hrs. prior to inoculation of the assay. Before inoculation, the broth cultures were centrifuged at 2,500 r.p.m. for 15 mins., washed with 10 ml. of physiological saline solution, recentrifuged, and made up in a saline solution to a turbidity of 70% against distilled water. Turbidity of the inoculum was determined in the same manner as turbidity of the assays.

The assay procedure employed eight dilution levels in triplicate for the standard curve and four dilution levels, in duplicate, for each sample. After inoculation with one drop of inoculum, the assay was incubated for 16 hrs. at 37°C. The results, determined turbidimetrically at 650 mμ. using filter PC-5 in a Coleman Universal spectrophotometer, were averaged for the four dilutions and again for the duplicates. The value thus obtained is reported.

Results

The moisture and nitrogen contents of the two glutens were as follows:

	Moisture	*Nitrogen (dry basis)*
Wheat gluten	67.9%	16.1%
Carob gluten	61.0%	16.3%

Results of the amino acid assays, in Table I, indicate a difference in the amino acid compositions of the two glutens. This is especially true for the basic amino acids, arginine, lysine, and histidine, which are found in larger proportions in the carob gluten and the heterocyclic

[4] Cultures were obtained from the Dept. of Biochemistry and Nutrition, Cornell University.

TABLE I

AMINO ACID CONTENTS OF WHEAT AND CAROB GLUTENS BY MICROBIOLOGICAL METHODS

Amino acid	Wheat gluten[1]	Carob gluten[1]	Organism	Medium
Glycine	2.8	4.1	C	II
Leucine	5.6	5.0	B	I
Threonine	2.1	2.6	B	I
Alanine	1.7	2.1	A	II
Isoleucine	3.6	3.2	B	I
Valine	3.5	3.5	B	I
Phenylalanine	4.4	2.7	C	I
Tyrosine	3.4	2.2	B	I
Cystine	2.2	1.2	C	II
Methionine	1.3	0.6	B	I
Glutamic acid	28.6	20.2	C	II
Aspartic acid	2.7	6.1	C	II
Arginine	3.3	12.8	B	I
Lysine	1.4	4.3	C	II
Histidine	1.9	2.5	B	I
Proline	10.7	2.6	C	II
Tryptophan	0.9	0.6	B	I

A—*Leuconostoc citrovorum*, 8081
B—*Streptococcus faecalis*
C—*Leuconostoc mesenteroides*, P-60
I—Stokes, *et al* (11)
II—Steele, *et al* (10)

[1] g./16.0 g. nitrogen.

TABLE II

COMPARISON OF WHEAT GLUTEN COMPOSITION WITH VALUES REPORTED IN THE LITERATURE

Amino acid[1]	Present investigation	Block and Bolling (4)	Padoa (7)	
Alanine	1.7	5	5.0	3.5[2]
Arginine	3.3	3.9	1.9	
Aspartic acid	2.7	10	9.6	
Cystine	2.2	1.7	1.4	
Glutamic acid	28.6	27	26.8	
Glycine	2.8	9	8.6	
Histidine	1.9	2.2	0.97	
Isoleucine	3.6	—	—	
Leucine	5.6	—	8.60	
Lysine	1.4	1.9	1.2	
Methionine	1.3	3	—	1.56[3]
Phenylalanine	4.4	5.5	4.08	
Proline	10.7	10	8.05	
Threonine	2.1	2.5	—	
Tryptophan	0.9	1.0	1.24	0.93[4]
Tyrosine	3.4	3.8	1.34	
Valine	3.5	—	3.27	

[1] g./16.0 g. nitrogen.
[2] Sauberlich and Baumann (9).
[3] Reisen, Schweigert, and Elvehjem (8).
[4] Greenhut, Schweigert, and Elvehjem (5).

amino acids, proline and tryptophan, present to a greater extent in wheat gluten.

A comparison of amino acid values found for wheat gluten with those previously reported in the literature (4), (5), (7), (8), (9) is given in Table II. In making this comparison, several factors should be borne in mind. Many of the values (4), (7) were determined by chemical analyses rather than by microbiological assay. Others (5), (8), (9) were obtained by microbiological assays with titration of acidity rather than turbidity as the measure of growth. Discrepancies among assays may also result from variations in raw material, poor recovery, and destruction or racemization of amino acids during hydrolysis. Poor agreement (low values) may be noted for aspartic acid, alanine, and glycine. Otherwise the values found by the procedures employed check well with those reported by other workers.

Discussion

The preparation of a material having the peculiar physical properties characteristic of gluten from a source other than wheat flour is of considerable interest in that it offers unique possibilities for fundamental studies on the factors responsible for these physical properties and the manner in which they may be modified by various treatments. That the formation of a glutinous structure in wheat flour dough is dependent on proper physical manipulation is generally recognized. Similar observations were made with the carob germ flour. Obtaining a strong, coherent gluten from this product required that the flour be sufficiently finely ground, that the proper amounts of water, time, and physical working be employed in preparation of the dough.

Gluten is by nature a heterogeneous mixture. Attempts to purify it by even rather mild treatments may greatly modify its properties. By comparing the compositions of glutens from different sources, some insight may be gained into which are the essential and which the nonessential constituents.

Microbiological methods for determination of amino acids cannot be regarded as perfected. Nevertheless these methods are sufficiently accurate to have become exceedingly valuable tools particularly for comparing the approximate compositions of proteins.

Since the amino acid composition of carob gluten differs quantitatively from that of wheat gluten it appears that a highly specific amino acid composition is not essential for a protein to exhibit glutinous properties. This does not, however, rule out the possibility that such properties reflect the presence of certain types of amino acid groupings with others acting as diluents having no specific influence on the physical properties.

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AN ELECTROPHORETIC ANALYSIS OF SOYBEAN PROTEIN[1, 2]

D. R. BRIGGS and ROBERT L. MANN[3]

ABSTRACT

Electrophoresis patterns for water extracts of defatted soybean meal, containing 95% of the total nitrogen, disclosed the presence of at least seven electrophoretically distinct proteins. "Glycinin," the globulin commonly considered to be the principal protein of soybeans, was found to be a mixture of components which constituted about 75% of the total soybean protein.

The composition of the globulin preparations, as shown by electrophoresis, varied considerably depending on the method of isolation. An electrophoretically homogeneous protein representing 60% of the globulin fraction was precipitated by cooling a water extract of the meal, the precipitation being enhanced by the addition of a small amount of calcium chloride. Its isoelectric point, as determined by microelectrophoresis, was pH 5.4. Solubility experiments with this protein fraction indicated it to be non-homogeneous by this criterion.

Osborne and Campbell (9) gave the name *glycinin* to that protein fraction which precipitated when a 10% sodium chloride extract of

[1] Manuscript received January 13, 1950.
Paper No. 2529, Scientific Journal Series, Minnesota Agricultural Experiment Station.
[2] Contents of this paper constitute a part of a thesis submitted by Robert L. Mann to the Graduate Faculty of the University of Minnesota in partial fulfillment of the requirements for the Doctor of Philosophy degree, June 1949.
[3] Present address: Eli Lilly and Company, Indianapolis, Indiana.

defatted soybean meal was dialyzed against water. They also reported the separation of three other proteins. Jones and Csonka (6) obtained five proteins by fractionation with ammonium sulfate. The fraction precipitable from a 10% sodium chloride extract at 55% saturation with ammonium sulfate resembled the glycinin of Osborne and Campbell. Its isoelectric point was pH 5.2. Earlier (2), glycinin prepared by the method of Osborne and Campbell was reported to have an isoelectric point of pH 4.7. Hartman and Cheng (3) prepared a "purified glycinin" and found the isoelectric point to be pH 5.02.

Smiley and Smith (1) showed that the nitrogen content of 16 samples of "glycinin" prepared by several methods ranged from 15.68% to 17.74%. McKinney, Sollars, and Setzkorn (8) have also recognized the fact that the composition of "glycinin" is dependent upon the method of preparation.

In view of the uncertainty which exists concerning the composition of whole soybean protein and of the anomalous behavior of glycinin when isolated by different methods, this investigation was made in an attempt to define more clearly this complicated protein system by subjecting it to electrophoretic analysis.

Materials and Methods

Materials. Wisconsin Manchu soybeans grown in 1946 at the University of Minnesota were used throughout the experimental work. Samples suitable for protein extraction were prepared by grinding the beans in a Wiley mill through a 0.5 mm. screen and removing the oil by extraction with petroleum ether (boiling range 30°–60°C.) in a Soxhlet extractor. The defatted meal was air dried and stored in stoppered bottles in a cold room. A typical fat-extracted meal sample showed the following percentage composition: moisture, 9.5; nitrogen, 7.26; ash, 5.29.

Extraction Method. The procedure used for extracting the protein from the oil-free meal was essentially that employed by Smith *et al.* (11). Defatted meal and a portion of the solvent were placed in a centrifuge bottle and mechanically shaken for 30 min., then centrifuged until the supernatant was clear. The type of extracting medium and the ratio of meal to solvent were varied during the investigation. Depending upon the nature of the experiment, either these extracts constituted the protein solutions studied, or the protein was precipitated by one of several methods and redispersed in an appropriate solvent.

Electrophoretic Analysis. Protein solutions to be analyzed electrophoretically were equilibrated by dialysis for at least four days at 4°C. against several changes of a suitable buffer—toluene being used to

inhibit bacterial growth. Electrophoresis of the solutions was observed in a Tiselius apparatus equipped with a Longsworth scanning device (7). On completion of a run a scanned photograph was taken. The field strength employed in all experiments was 5.5 volts/cm. and unless otherwise noted the time of each run was 6,440 sec. Since, for purposes of comparison, it is necessary in most cases to superimpose one electrophoretic pattern on another, tracings of projections of these plates are shown in this report of the work. In each figure is shown a scale equivalent to 1 cm. in the electrophoresis cell.

Results

Electrophoresis of The Water Extractable Protein. Extraction of 3 g. of meal with 100 ml. of water resulted in the dispersion of 95.1% of the total nitrogen present. The solution obtained after centrifuging, although slightly cloudy, was sufficiently clear for electrophoretic analysis. Three extracts were prepared in this manner. Two were dialyzed against phosphate buffers, one at pH 7.6, 0.1 ionic strength, the other at pH 6.6, 0.1 ionic strength. The third extract was equilibrated against veronal buffer at pH 9.0 and 0.09 ionic strength.

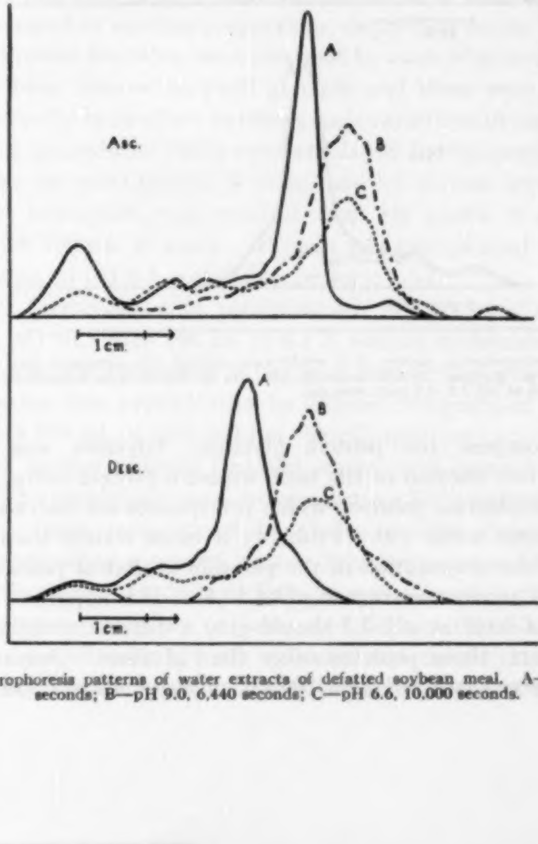

Fig. 1. Electrophoresis patterns of water extracts of defatted soybean meal. A—pH 7.16, 6,440 seconds; B—pH 9.0, 6,440 seconds; C—pH 6.6, 10,000 seconds.

Results of electrophoresis of these preparations, each being adjusted to approximately a 1% protein concentration, are shown in Fig. 1.

These patterns reveal that while there exist at least seven electrophoretically distinct proteins in the extract, approximately 75% of the total protein is present as a fast moving fraction which appears to consist of three electrophoretically different proteins.

Since these extracts are complicated mixtures, it was desired to determine, if possible, which of the electrophoretically distinguishable

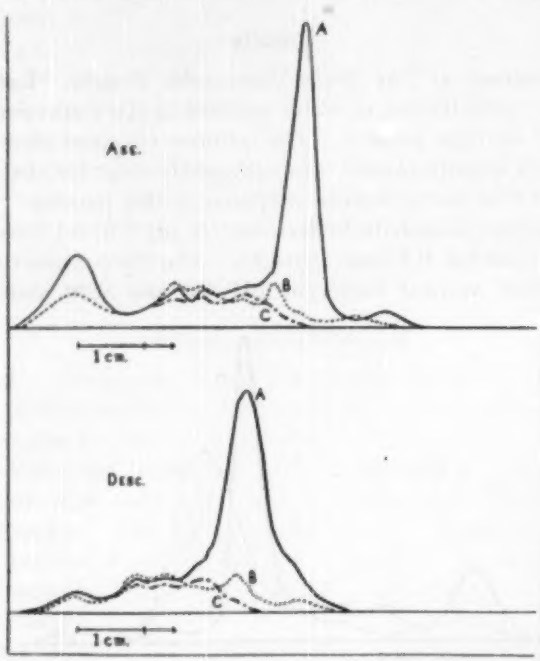

FIG. 2. Electrophoresis pattern of a whole water extract (A), compared with water extracts after elimination of "glycinin" by acid extraction (B), and by dialysis of an aqueous extract (C). The solutions were run at pH 7.6, 0.1 ionic strength.

fractions compose the protein glycinin. Glycinin was originally defined as that fraction of the total soybean protein extractable with 10% sodium chloride solution which precipitates on dialysis of a meal extract against water (9). Probably a more widely used alternate method for the preparation of the globulin is that of precipitation by adjusting an aqueous extract to pH 4.5 (4). It is apparent, then, that extraction of meal at pH 4.5 should give a dispersion containing, for the most part, those proteins other than glycinin. Accordingly, 16 g. of meal were extracted with 100 ml. of water maintained at pH 4.5

with acetic acid. This extract was dialyzed against phosphate buffer of 0.1 ionic strength and pH 7.6.

For comparison 16 g. of meal were extracted with 100 ml. of water and the extract dialyzed against running distilled water in the cold until precipitation was complete. The precipitate was removed by centrifugation and the supernatant liquid dialyzed against phosphate buffer of 0.1 ionic strength at pH 7.6.

The electrophoretic analyses of these two solutions should indicate which proteins are precipitated by acidification or by dialysis, i.e., the so-called glycinin fraction. The patterns are compared with that of a complete water extract of meal in Fig. 2.

While the patterns shown in Fig. 2 cannot be considered as yielding an accurately quantitative comparison, it is, nevertheless, evident that the globulin fraction of the soybean protein is identified with the large faster moving peak (or peaks) of the electrophoresis pattern obtained with the complete water extract of the meal.

Electrophoretic Analysis of "Glycinin" Prepared by Several Methods. Since the patterns of Fig. 2 indicate that glycinin may consist of more than a single component, the reported variation in composition and properties of this material may well be due to the several components being precipitated in varying proportions, depending on the method of isolation. Protein fractions were prepared by each of several methods which have been claimed to yield glycinin and these were examined electrophoretically in an effort to detect such variations in composition. The following procedures which were employed for the preparation of these samples are quite typical of those used by various investigators.

A. Water extraction, acid precipitation: Six grams of meal were extracted with 100 ml. of water. Protein was precipitated by adjusting the solution to pH 4.5 with dilute sulfuric acid.

B. Alkali extraction, acid precipitation: Six grams of meal were extracted at pH 10.5 with 100 ml. of 0.1 *N* sodium hydroxide solution. The protein was precipitated as in (A) with sulfuric acid at pH 4.5.

C. Salt extraction, precipitation by dialysis: Six grams of meal were extracted with 100 ml. of 10% sodium chloride solution and the extract was dialyzed against running distilled water until precipitation was complete. The precipitate was redispersed in 10% sodium chloride solution and again precipitated by dialysis. The dispersion and dialysis were repeated a second time. This preparation should correspond to the original glycinin of Osborne and Campbell (9).

D. Preparation according to Jones and Csonka (6): Six grams of meal were extracted with 100 ml. of water and the extract dialyzed against running distilled water. The precipitate obtained was taken

up in 10% sodium chloride solution and the glycinin precipitated by the addition of ammonium sulfate to 55% saturation.

The following amounts of protein, based on the per cent of total nitrogen extracted, were obtained as "glycinin" by each method: A, 71.6%; B, 70.0%; C, 67.3%; D, 59.0%. Each of the above preparations was dissolved in buffer so as to yield an approximately 1% protein solution and all were equilibrated against phosphate buffer of 0.1 ionic strength at pH 7.6 and examined in the electrophoresis apparatus. The Tiselius patterns are shown in Fig. 3.

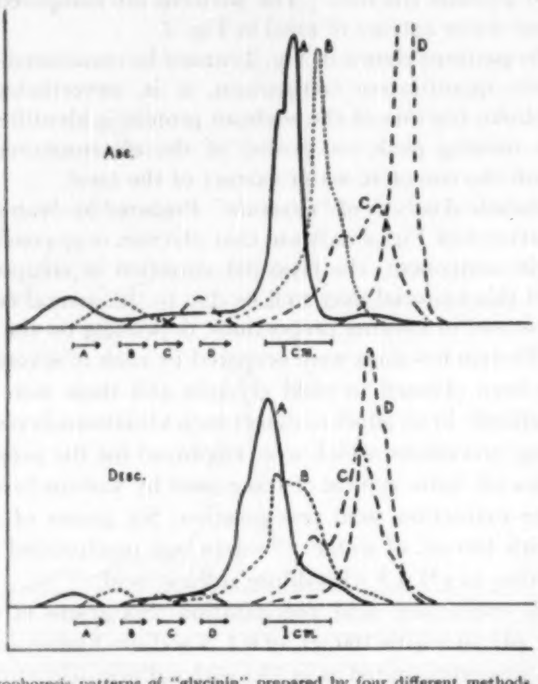

FIG. 3. Electrophoresis patterns of "glycinin" prepared by four different methods. A—method A; B—method B; C—method C; D—method D. The solutions were run at pH 7.6, 0.1 ionic strength.

The variability of "glycinin" preparations is demonstrated by these patterns. It is evident that the globulin prepared according to the method of Osborne and Campbell is electrophoretically inhomogeneous and that none of the other preparations which have been identified by the name, glycinin, is electrophoretically homogeneous or identical with the preparation of Osborne and Campbell.

The ammonium sulfate fractionation of a meal extract to give five proteins, as reported by Jones and Csonka (6), was repeated and all the fractions were found to be electrophoretically heterogeneous.

Isolation of an Electrophoretically Homogeneous Soybean Protein. During some preliminary experiments it was observed that when a concentrated meal extract was cooled to 0° C. a precipitate formed which would redisperse on warming the solution again to room temperature. This precipitated material could be removed by centrifugation in the cold and, by electrophoretic comparison of aqueous extracts before and after its removal, it was shown to constitute a part of the "glycinin" fraction. In addition, the patterns gave some indication that the precipitate was composed primarily of only one of the proteins belonging to this group. This offered a clue to a possible means of separating a single protein component from a water extract.

The method used for the preparation of this cold-precipitable protein was as follows: Ten to 20 g. of meal were extracted with 100 ml. of water. The high ratio of meal to solvent was necessary since little or no precipitation occurred on cooling the aqueous extract unless the total protein concentration was somewhat greater than 1%. A centrifuge tube containing the extract was placed in ice water and allowed to stand several hours. The cloudy suspension which formed was centrifuged in the cold and the supernatant discarded. The residue was an amber colored, translucent syrup which dispersed very readily in a small quantity of distilled water. An aqueous solution of this cold-precipitated protein was clarified by centrifuging at room temperature and dialyzed against distilled water until complete precipitation of the protein resulted. The aqueous suspension of protein was either dried *in vacuo* from the frozen state or the protein was removed by centrifugation, dehydrated by washing with methyl alcohol, and dried with ether. In either case the product was a white powder which was soluble in 2% sodium chloride.

Three solutions containing this protein in a concentration of 1% were prepared with the following buffers: potassium chloride-hydrochloric acid buffer, 0.1 ionic strength, pH 2.9; phosphate buffer, 0.1 ionic strength, pH 7.3; phosphate buffer, 0.1 ionic strength, pH 7.6. The patterns obtained from the electrophoretic analysis of these solutions are shown in Fig. 4. No distinguishable electrophoretic inhomogeneity of this protein fraction is apparent.

The next step in studying this apparently homogeneous fraction was to determine to what extent it occurs in a water extract of meal. In a series of extracts having a total protein concentration varying from 2% to 4%, approximately 30% of the total protein precipitated on cooling. In this concentration range, the amount precipitated appeared to be independent of the total protein concentration.

Smith, Circle, and Brother (11) have extensively studied the peptizability of soybean protein by neutral salts and shown that in dilute

solutions there is a sharp minimum in the peptization curve which varies in degree with the kind of salt used. They found that the curves could be fairly well reproduced by the alternate procedure of starting with a water extract and precipitating the protein by the addition of increasing quantities of salt. In the present study, aqueous solutions of the cold-precipitated protein were observed to be extremely sensitive to precipitation by the addition of neutral salts and this suggested the possibility that more of the cold-precipitable protein

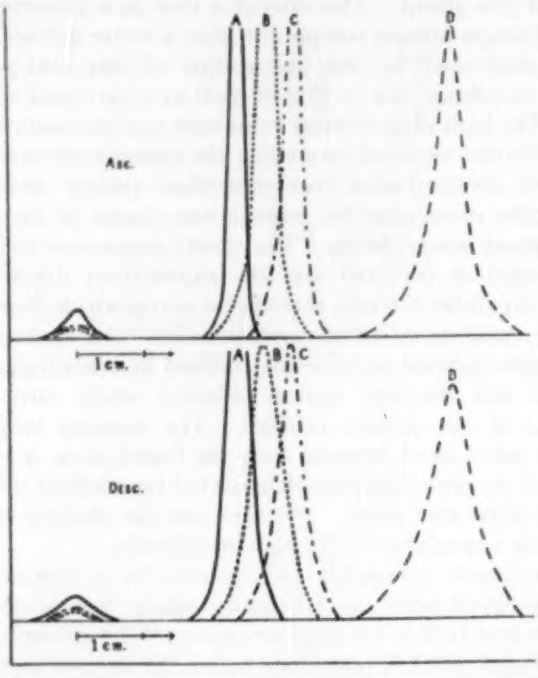

FIG. 4. Electrophoresis patterns of cold-precipitated protein run at different pH values. A—pH 2.9, 0.1 ionic strength, 6,440 seconds; B—pH 7.3, 0.1 ionic strength, 6,440 seconds; C—pH 7.6, 0.1 ionic strength, 6,440 seconds; D—pH 7.3, 0.1 ionic strength, 12,630 seconds.

might be obtained from the cooled aqueous extracts by such addition of salts. Because of the coincidence of its precipitation and peptization curves as indicated in the data of Smith, *et al.* (11), calcium chloride was chosen as the salt to be used.

Fifteen grams of meal were extracted with 100 ml. of water. The extract was cooled and the cold-precipitated protein removed by centrifugation. After warming the extract again to room temperature, calcium chloride solution was added in an amount insufficient to

bring about precipitation at room temperature. The pH was adjusted back to 6.5 (pH of the water extract) by the addition of a minute quantity of sodium hydroxide solution. The solution was then cooled again. More precipitate formed and was removed by centrifugation. This process was repeated until it became impossible to add any further calcium chloride at room temperature without precipitation. At this point it was assumed that all the cold-precipitable protein had been removed. The original cold-precipitated material plus that precipitated by adding the salt and cooling comprised 44.1% of the total soybean protein. The electrophoretic behavior of those fractions obtained on cooling after calcium chloride addition was identical in every respect with the fraction that precipitated initially from a water extract in the cold. It may be concluded that at least 44% of the total protein extractable from soybean meal by water can be isolated as an electrophoretically homogeneous material. If it is assumed that proteins precipitated by dialysis (globulins) comprise 75% of the total protein, then the cold-precipitable fraction constitutes about 60% of this fraction.

Two rather interesting possibilities were suggested by these experiments. First, Smith, *et al.* (11) found that the minimum point on the sodium chloride peptization curve showed that 48.8% of the total water extractable nitrogen was not extracted at the corresponding salt concentration. The agreement between this figure and the value 44.1% for the apparent total cold-precipitable protein obtained in the present study appears to be more than coincidental. It may be postulated that the precipitating effect of the sodium chloride is specific for the cold-precipitable protein. Some justification for this idea is found in the observation that an aqueous extract, after removal of this protein fraction, shows no precipitation on the addition of sodium chloride in any quantity. Further, the protein precipitated by sodium chloride from a water extract of meal showed electrophoretic properties identical with the cold-precipitated protein.

Second, as in the case of sodium chloride, the addition of the first increments of calcium chloride apparently causes precipitation of the cold-precipitable protein alone. However, when calcium chloride is added in an amount which will yield maximum precipitation at room temperature, the amount of protein that is precipitated represents 76% of the total extracted by water (11)—a value corresponding favorably with the percentage of total globulin components present in an extract. To determine if calcium chloride precipitates only the globulin fraction, electrophoretic patterns of an extract before and after maximum calcium chloride precipitation were made. The salt precipitation was carried out on an extract of 6.5 g. of meal with 100 ml.

of water by adding calcium chloride in small quantities until no more precipitation occurred. The coagulated protein was removed by centrifugation and a nitrogen analysis of the supernatant indicated 73.2% of the nitrogen present in the original extract had been eliminated by precipitation. The electrophoresis patterns for the proteins of the original extract and of those not precipitable by calcium chloride are compared in Fig. 5. The supposition that calcium chloride, at that concentration where it shows its maximum capacity to precipitate the

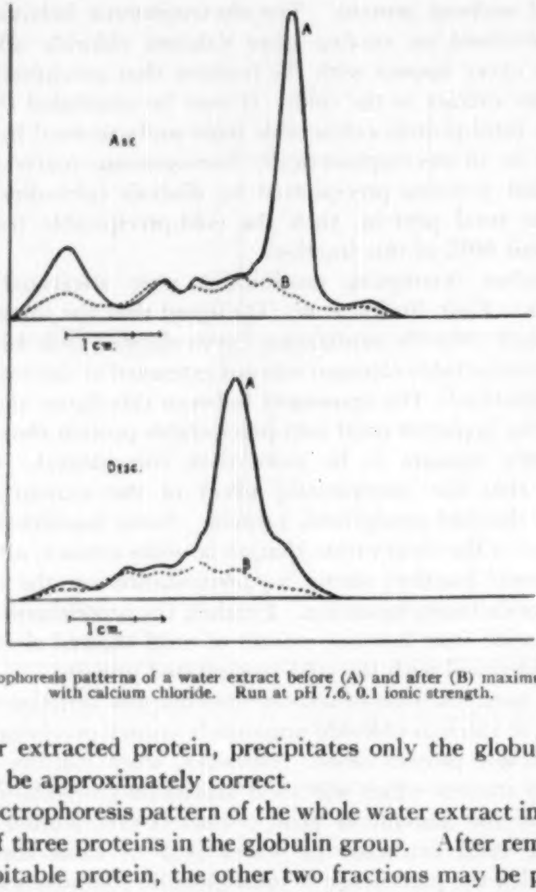

FIG. 5. Electrophoresis patterns of a water extract before (A) and after (B) maximum precipitation with calcium chloride. Run at pH 7.6, 0.1 ionic strength.

total water extracted protein, precipitates only the globulin fraction appears to be approximately correct.

The electrophoresis pattern of the whole water extract indicated the presence of three proteins in the globulin group. After removal of the cold-precipitable protein, the other two fractions may be precipitated by the further addition of calcium chloride. All attempts to fractionate these two components with calcium chloride resulted only in precipitation of mixtures of the two. Apparently both are coagulated by calcium chloride with comparable facility. The isoelectric point of

this mixture as determined by the method of microelectrophoresis was pH 4.8.

Some Properties of the Electrophoretically Homogeneous Protein. A detailed investigation of the optimal conditions for obtaining this protein by cooling an extract has not been carried out. It seems, however, that water is the best extracting medium. No cold precipitation will take place from a 0.85*M* sodium chloride solution, for example. The extent to which the presence of added salts affects the precipitation is not known. On the basis of purely qualitative turbidity experiments, the amount of precipitate formed is dependent on both pH and salt concentration. At pH 7.6 little cloudiness was observed at 0.2 ionic strength, but normal precipitation apparently took place at 0.1 ionic strength. At pH 6.0, however, cold precipitation occurred readily at 0.2 ionic strength.

A partial analysis of the protein showed that it contained 17.17% nitrogen, 0.73% sulfur, 0.05% phosphorus, and 0.10% ash on a dry-matter basis.

As a further criterion of purity, the solubility method (5) was applied to the electrophoretically homogeneous protein. The wet curd which resulted after dialysis of a water dispersion of the protein was suspended in a small quantity of a suitable solvent. The suspension was allowed to equilibrate with a large volume of the solvent by dialysis. Two solvents were used—1.8% sodium chloride buffered at pH 5.9 with potassium phosphate salts, 0.1 ionic strength, and phosphate buffer at pH 7.0, 0.1 ionic strength. Solubilities were determined at 5°C. and 20°C., respectively. Varying amounts of the equilibrated suspension were made to volume with the solvent and nitrogen was determined on aliquots. The solutions were rocked in a mechanical rocker until equilibrium was reached, filtered, and nitrogen was determined on aliquots of the filtrate. Fig. 6 and Fig. 7 show

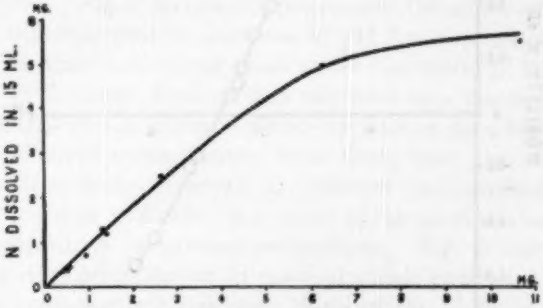

FIG. 6. Solubility of cold-precipitated protein in 1.8% sodium chloride, pH 5.9 at 5°C.

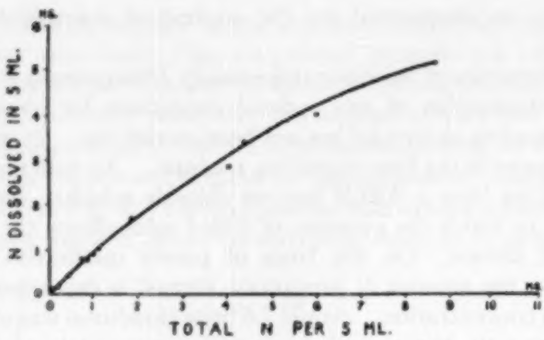

FIG. 7. Solubility of cold-precipitated protein in phosphate buffer, pH 7.0, 0.1 ionic strength at 20°C.

plots of total nitrogen present against nitrogen dissolved by the two solvents.

The solubility of this protein is not independent of the solid phase present and the protein cannot, therefore, be considered to be truly homogeneous.

The isoelectric point of the cold-precipitable protein was determined by the method of microelectrophoresis using a horizontal cell of the type designed by Briggs (1). Mobilities were measured by observing the movement of protein-coated quartz particles suspended in

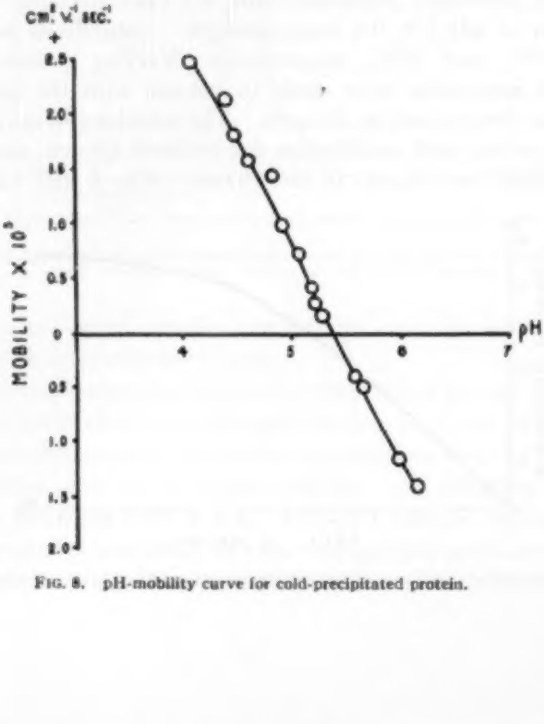

FIG. 8. pH-mobility curve for cold-precipitated protein.

acetate buffers of 0.01 ionic strength. The isoelectric point is at pH 5.4 as indicated by the pH-mobility curve shown in Fig. 8.

The Effects of Varying the Ratio of Meal to Water During the Extraction of Protein. Water was shown to extract 95% of the total nitrogen from meal when a ratio of 3 g. of meal to 100 ml. of water was used. In the work involving isolation of the cold-precipitable protein it was necessary to use rather high concentrations of protein which were most readily obtained by increasing the ratio of meal to water during the extraction process. However, at a meal to water ratio of 20 to 100, only about 50% of the nitrogen could be extracted. The question arose as to whether or not this 50% extract contained all the proteins in the same proportions as in the 95% extract. There was the possibility that in the 50% extract some of the proteins were preferentially retained in the meal. The answer to this question was obtained by making two extractions, one at a meal to water ratio of 3 to 100, which extracted 95% of the nitrogen, and one at a meal to water ratio of 20 to 100, which extracted 54.6% of the nitrogen. Both extracts after dialysis against buffer were adjusted by dilution with buffer solution to the same nitrogen content and examined electrophoretically. The patterns for these extracts were identical, showing the proteins to be present in the same ratio regardless of the amount extracted. Thus, the comparison of information obtained from a 50% extract, for example, with a 95% extract is justifiable.

Discussion

Since water will extract over 95% of the total nitrogen present in soybeans, the electrophoresis patterns of such extracts present a rather complete picture of soybean protein. The Tiselius patterns of aqueous extracts adjusted to different pH values do not show significant differences other than a separation of the components to a greater or lesser degree. Fig. 1 reveals the presence in the water extract of at least seven different protein fractions at pH 7.6 in phosphate buffer. Planimeter measurement of the areas under the curves of Fig. 2 shows that about 75% of the total protein migrates as a fraction which is identified as globulin, a material which appears to be a mixture of at least three different components. It is likely that the variation in composition frequently observed in different preparations of "glycinin," i.e., soybean globulins, is a result of the precipitation of these globulin components in varying proportions. Fig. 3 also indicates that, during acid precipitation in particular (see pattern A), some of the protein components other than those of the globulin group are carried down and thus contribute to the variability in composition of the preparations compared. The most nearly homogeneous of the

preparations studied appears to be that obtained by the method of Jones and Csonka who reported its isoelectric point to be pH 5.2 (6), a value close to that of the electrophoretically homogeneous protein reported in this paper.

The effect of the presence of neutral salts on the solubility of the various soybean proteins is of considerable interest in connection with their isolation. Although sodium chloride and calcium chloride were the only salts used in this investigation, it is probable that their precipitating action is typical of the effects shown by other salts of monovalent and divalent cations respectively. That is, extraction of meal with the salts of monovalent cations at concentrations near to that of minimum peptizing capacity would not, in general, be expected to result in the dispersion of the cold-precipitable component of soybean globulin; and extraction with salts of divalent cations at corresponding concentrations would leave the entire globulin group undispersed.

No adequate explanation for the water peptizability of soybean proteins has been attempted. Such an explanation must certainly require an understanding of the action of salts on the dispersibility of the proteins in general. The precipitate, obtained on cooling an aqueous meal extract, is very soluble in water and its aqueous solutions are extremely sensitive to the addition of salts and to the removal of salts by dialysis. In view of these findings it is believed that a further investigation of the solubility properties of this fraction could contribute considerable information which would be useful in any attempt to devise an explanation of the water and salt solution peptizability of the globulins present in soybeans. The use of this system for such a study would be advantageous in that it is undoubtedly less complex than any previously described preparation of "glycinin."

It must be concluded that, on the basis of its present definition, the use of the term "glycinin" to designate any single component of the soybean protein is incompatible with the electrophoretic evidence presented in this study. The preparation of Osborne & Campbell, to which this name was originally applied, is obviously a mixture of electrophoretically distinguishable components, as is the case also with all other "glycinin" preparations studied; and the various preparations are not identical with respect to their composition ratios of these electrophoretically distinguishable components. It would appear wise to designate only the preparation of Osborne & Campbell by this term, at least until further study may indicate clearly how the term may best be redefined to designate some predominant and reasonably homogeneous globulin fraction of the soybean protein. Perhaps the cold precipitable globulin, as partially characterized in this study, may

logically, and after further characterization, inherit the name. Considerable further study of the other protein fractions of the soybean must be made, however, before any redefinition of the term should be settled upon.

Acknowledgments

The authors are indebted to the Central Soya Company, Inc., Decatur, Indiana, who provided the fellowship during the tenure of which part of this research was conducted.

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EFFECTS OF SOLVENT AND HEAT TREATMENTS ON SOYBEAN PROTEINS AS EVIDENCED BY ELECTROPHORETIC ANALYSIS[1,2]

ROBERT L. MANN[3] and D. R. BRIGGS

ABSTRACT

The effects of heat and of methanol and ethanol extraction of soybean meal on the peptizability of the soy proteins were studied electrophoretically. Hot and cold methanol or ethanol extraction of the meal reduced the subsequent extractability with water or salt solutions of all the protein components but the effect was most pronounced on the globulin components. The hot extractions were the more effective. Heating aqueous extracts of soybean meal resulted in the precipitation of protein in varying amounts, the quantity being precipitated increased with increase in temperature and with length of the heating period. This precipitation was probably the result of a heat accelerated interaction of the protein components and involved primarily those protein fractions other than the globulins. While the isolated globulins alone were unaffected in their dispersibility by heat treatment, they became increasingly more nondispersible when heated in the presence of the other protein components of the soybean extract.

The commercial value of fat-free soybean meal is dependent in part upon the extractability of the proteins which it contains. Since some methods of processing the bean subject it to contact with organic solvents and heat, it is apparent that a knowledge of the effects of these conditions on the extractability of the protein is extremely important.

For the most part, previous investigations have involved studies of the effects of heat and of methanol or ethanol extraction (1, 2, 4, 11). Although the data thus far accumulated are somewhat variable, they indicate that heat is the more important factor in reducing the amount of protein that can be subsequently extracted from soybean meal, and the effect becomes more pronounced as the moisture content of the meal is increased.

In these investigations, the so-called "denaturing" action of these agents was determined primarily by following changes in the peptizability of the protein in water, salt solutions, or alkali solutions. Such a procedure, of course, gives no information as to which components of the whole protein have become less peptizable after treatment

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[1] Paper No. 2530, Scientific Journal Series, Minnesota Agricultural Experiment Station. (From the Division of Agricultural Biochemistry, University of Minnesota, St. Paul 1, Minnesota.)

[2] Contents of this paper constitute a part of a thesis submitted by Robert L. Mann to the Graduate Faculty of the University of Minnesota in partial fulfillment of the requirements for the Doctor of Philosophy degree, June 1949.

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nor does it indicate the nature of the changes, if any, which may be taking place in the protein molecules as a result of such treatment. It seemed that considerable information might be gained by using the method of electrophoresis in studying the effects of heat, methyl alcohol, and ethyl alcohol on soybean protein.

Materials and Methods

Materials. Wisconsin Manchu soybeans grown in 1946 at the University of Minnesota were used. The beans were ground in a Wiley mill to pass a 0.5 mm screen and the oil was removed by Soxhlet extraction with petroleum ether (boiling range 30°–60°C.). The defatted meal was air dried and stored in stoppered bottles at approximately 5°C.

Extraction Method. The procedure used for extracting the protein with water or salt solutions was similar to that described by Smith *et al.* (9). The meal and a portion of the solvent were placed in a centrifuge bottle and mechanically shaken for 30 min., then centrifuged until the supernatant was clear. The amount of nitrogen extracted was determined by micro-Kjeldahl analysis of aliquots of the supernatant solution.

Alcohol treatment used prior to aqueous extraction. Defatted meal was extracted with hot or cold absolute methanol or 95% ethanol. For the hot extraction a continuous type Soxhlet extractor was used so that the temperature of the solvent in contact with the meal was 60°C. in the case of methanol and 73°C. with ethanol. The cold extractions were carried out at 20°C. in a Soxhlet extractor modified so that the solvent passed through a water-cooled coil before coming in contact with the meal. In most cases 1 g. of meal was extracted with the solvent for a definite length of time, removed from the extractor, and allowed to dry in air. The entire residue was then used for the aqueous extraction of protein.

Heat Treatment. A solution to be heated was placed in a test tube equipped with a condenser and immersed in an oil bath automatically maintained at the desired temperature. The time and the temperature of heat have been varied during the investigation.

Electrophoretic Analysis. Protein solutions to be analyzed electrophoretically were equilibrated by dialysis for at least four days at 4°C. against several changes of phosphate buffer of pH 7.6 and 0.1 ionic strength. Toluene was used to inhibit bacterial growth. Electrophoresis of the solutions was observed in a Tiselius apparatus equipped with a Longsworth scanning device (7). On completion of a run a scanned photograph was taken. The field strength employed was 5.5 volts/cm. and the time of each run was 6,440 seconds. The Tiselius

patterns are shown in the figures as traced outlines in order to allow for more convenient comparisons. In each figure is shown a scale representing 1 cm. in the electrophoresis cell.

Results

The Effect of Methanol and Ethanol. One gram portions of air-dried meal were extracted with hot or cold methyl or ethyl alcohol. After again drying in air each of the samples was extracted with 40 ml. of water and the amount of nitrogen in the extract was determined. The results of the experiment are shown in Table I. Hot solvent is

TABLE I

EFFECT OF SOLVENT EXTRACTION OF SOYBEAN MEAL ON THE DISPERSIBILITY OF THE PROTEIN IN WATER

Extraction Solvent	*Length of Solvent Extraction* *hrs.*	*Nitrogen Extracted by Water* %
None		89.0
Cold methanol (20°C.)	9.5	64.9
Cold methanol (20°C.)	50.0	57.7
Cold ethanol (20°C.)	9.5	60.0
Hot methanol (60°C.)	9.5	18.2
Hot ethanol (73°C.)	9.5	18.0

seen to decrease the subsequent water extractability of the proteins very markedly. Cold solvent is less effective in this regard but its effectiveness increases with the time of contact with the meal. Attempts were also made to extract the nitrogen from hot methanol or ethanol-extracted meal with sodium chloride solutions ranging in concentration from 0.02 *M* to 5 *M*. In no instance could more than 18% of the total nitrogen be extracted.

Electrophoresis patterns obtained with water extracts of hot and cold methanol extracted meals are compared in Fig. 1 with the pattern from a water extract of untreated meal. It is evdient from these patterns that the globulin fraction of the total soy protein (3) is the fraction whose water extractability is decreased most markedly by the preliminary treatment of the meal with alcohol. Hot alcohol is most effective in this regard.

The Effect of Heat. Since the object of this experiment was to determine the direct effect of heat on the soybean proteins and not on their extractability, it was necessary to confine the heating experiment to aqueous extracts of untreated (fat extracted) meal. Therefore, the results may not be entirely comparable with the information obtained by other workers who heated the meal and then determined the peptizability of the protein with various solvents. However, it is presumed that the proteins affected and the nature of the effect should, in general, be the same in both cases.

Preliminary experiments showed that precipitation of protein occurred when an aqueous extract of meal was heated. On the basis of this observation the following possible factors in the phenomenon were investigated: (a) the dependence of the precipitation on temperature, (b) its dependence on the length of time of heating, (c) the effect that the precipitation by heating might have on the amount of electrophoretically homogeneous protein which precipitates upon cooling (3), (d) which proteins are precipitated by heating, and (e) the changes

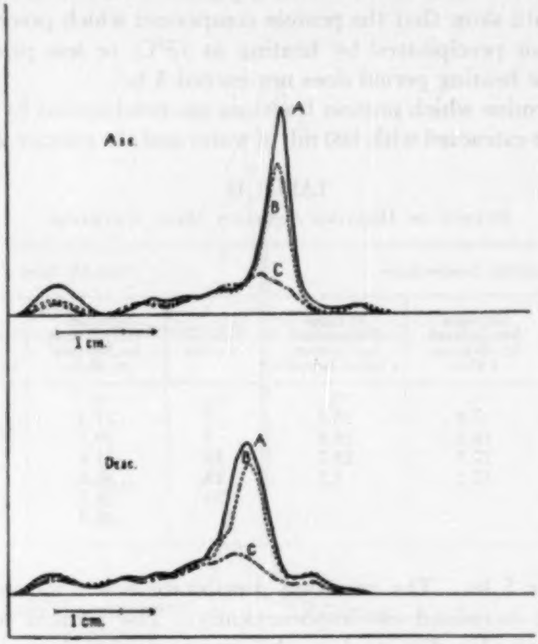

FIG. 1. Electrophoresis patterns, A—of the total protein extracted by 100 ml. of water from 3 g. of meal, B—of the total protein extracted by 100 ml. of water from 4 g. of meal previously extracted by methanol at 20°C., C—of the total protein extracted by 100 ml. of water from 15 g. of meal previously extracted by methanol at 60 C.

taking place in the electrophoretic properties of the protein molecules as a result of heat treatments.

Information concerning the first three problems was obtained from the following experiment. Twenty grams of meal were extracted with 100 ml. of water and a 10 ml. aliquot of the centrifuged extract was placed in each of a series of test tubes. Some of these tubes were heated for a two-hour period at 45°, 60°, 75°, and 90°C. respectively. The rest were heated at 75°C. for varying lengths of time from 2 hr. to 30 hr. At the completion of each heating period the precipitated

protein was removed by centrifugation and the per cent of total nitrogen which was removed from solution was determined. In none of the tubes did the pH of the solution drop more than 0.1–0.2 units below that of the original extract which was at pH 6.5. The tubes were then placed in ice water in a cold room until the cold precipitation of protein (a globulin fraction (3) is precipitated under these conditions) was complete. This precipitate was also removed by centrifugation and the percent of total (original) nitrogen lost by cold precipitation was determined. The results of the experiment are shown in Table II.

These data show that the protein component which precipitates on cooling is not precipitated by heating at 75°C. or less provided the length of the heating period does not exceed 5 hr.

To determine which protein fractions are precipitated by heat, 5 g. of meal were extracted with 100 ml. of water and the extract was heated

TABLE II

EFFECT OF HEATING AQUEOUS MEAL EXTRACTS

Variable Temperature			Variable Time		
Temperature of Heating	Nitrogen Precipitated by Heating 2 Hrs.	Nitrogen Precipitated by Cooling Heated Samples	Time of Heating	Nitrogen Precipitated by Heating at 75 C.	Nitrogen Precipitated by Cooling Heated Samples
C.	%	%	hrs.	%	%
45°	7.0	28.3	2	27.3	28.9
60°	16.3	28.8	5	29.3	28.9
75°	27.5	28.7	10	34.4	23.5
90°	52.2	3.5	15	36.6	22.2
			20	38.2	19 9
				40.3	19.6

at 75°C. for 5 hr. The resulting precipitate was removed and the supernatant examined electrophoretically. The protein which precipitated was also dispersed in the standard phosphate buffer and examined electrophoretically. These patterns are shown in Fig. 2.

In a previous paper (3) it was shown that electrophoretic patternsof water extracts of meal indicated the presence of at least seven different components, three of which migrated in a group which constituted the globulin fraction. One effect of the heating becomes strikingly apparent, the precipitation by heat involves, primarily, those proteins other than globulin. The mobilities of the globulin components do not appear to be altered by the treatment. It is also seen that while, in an ordinary water extract, those proteins which are precipitated by heating show up as at least three fairly distinct peaks in the electrophoresis patterns, the pattern obtained for the heat precipitated material shows a single, fairly symmetrical peak.

Since here the globulin fraction of soybean protein is apparently not involved, it was believed that a better picture of the effect of heat could be obtained if the experiments were conducted on extracts of meal made at pH 4.5, which have been shown (3) to contain all the proteins in their usual relative proportions except the globulins which are present in a greatly diminished proportion. An observation made during preliminary experiments was that actual precipitation on heating did not occur if the aqueous protein extract was first buffered with

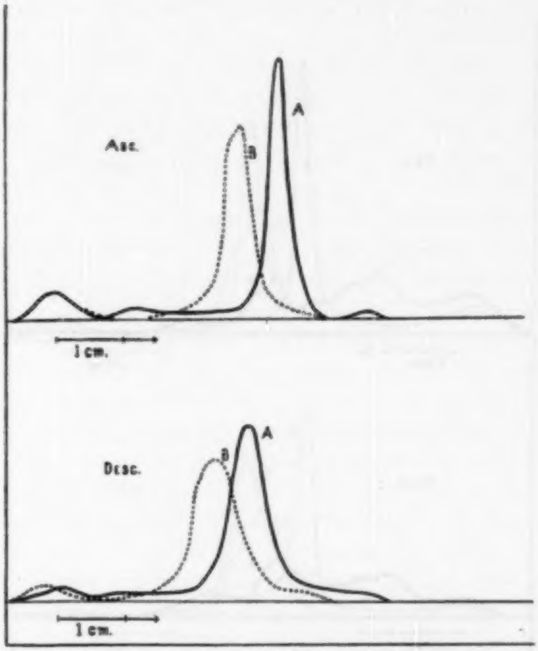

FIG. 2. Electrophoresis patterns. A—of a water extract of meal after removal of protein precipitated by heating 5 hrs. at 75°C., B—of the protein precipitated by heating a water extract of meal.

phosphate buffer at pH 7.6, 0.1 ionic strength and this proved to be useful in this connection.

Accordingly, 50 g. of meal were extracted with 200 ml. of water maintained at pH 4.5 with acetic acid. Part of this extract was dialyzed against buffer without heating. Another portion was buffered at pH 7.6 and heated in the oil bath at 75°C. for 2 hr. after which it was also dialyzed against buffer. No precipitation occurred in either portion. Both solutions were examined electrophoretically and the patterns obtained are shown in Fig. 3.

The change which occurs in the mobilities of these proteins cannot definitely be interpreted; but for the present, it may be considered as involving an interaction of these constituents under the influence of heat. The progress of this interaction could be followed by observing the electrophoresis patterns of extracts heated at 60°C. for 2 hr., for example. At this temperature and time the interaction was not complete, but part of the protein had been converted into a component migrating as a peak corresponding to the one shown for the heated sample in Fig. 3.

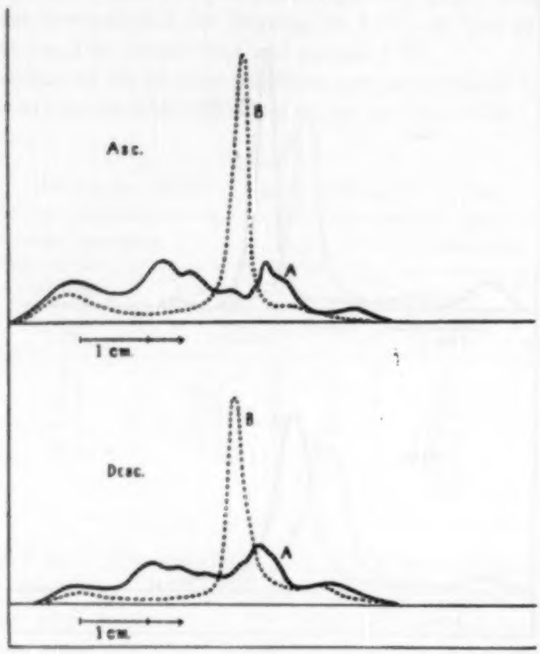

FIG. 3. Electrophoresis patterns. A—of an extract of meal made at pH 4.5. B—of the extract after heating.

As shown earlier (3), an aqueous extract of meal contains the non-globulin constituents to the extent of approximately 25% of the total protein. It is seen in Table II, however, that more protein precipitates upon heating at temperatures above 75°C., or at 75°C. when heated more than 5 hr., than can be accounted for by this 25% alone. This suggested the possibility of interaction involving, also, some of the protein of the globulin fraction. Such a reaction is implied, too, by the patterns of Fig. 3 where it is seen that the small globulin peak present in the native extract disappears on heating. In an attempt to

verify this supposition 25 g. of meal were extracted with 100 ml. of water at pH 4.5 and the extract was heated unbuffered at 75°C. for 2 hr. The resulting precipitate was removed and dispersed in buffer at pH 7.6, 0.1 ionic strength. This dispersion was diluted with an equal portion of a 1% solution of cold-precipitated protein (a globulin fraction). The mixture was examined electrophoretically before and after heating at 75°C. for 5 hr. The patterns are shown in Fig. 4.

The indication is that the minor protein components after interacting among themselves will undergo further interaction upon heating

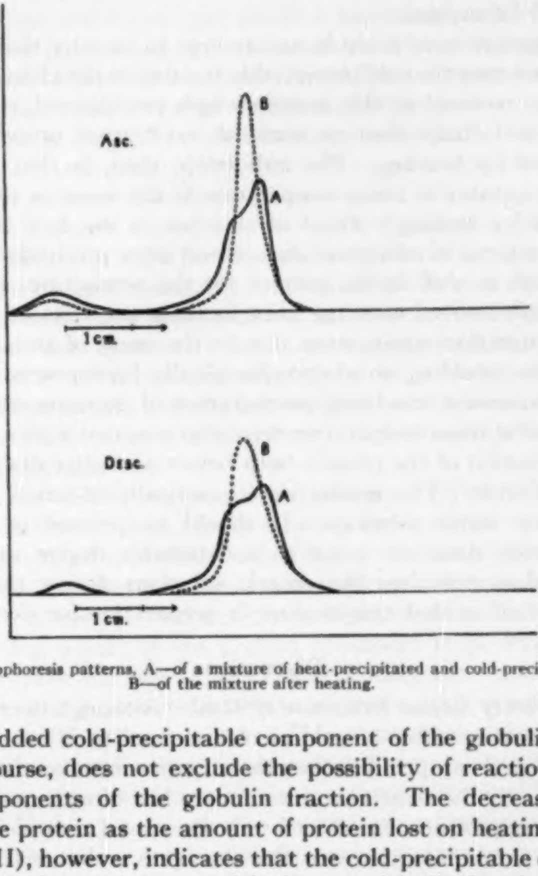

FIG. 4. Electrophoresis patterns, A—of a mixture of heat-precipitated and cold-precipitated protein, B—of the mixture after heating.

with the added cold-precipitable component of the globulin fraction. This, of course, does not exclude the possibility of reaction with the other components of the globulin fraction. The decrease in cold-precipitable protein as the amount of protein lost on heating increases (see TableII), however, indicates that the cold-precipitable component is among the first to interact with the non-globulin fractions as these undergo heat denaturation.

A rather interesting observation was made that is closely related to these heating effects. It was noted than an aqueous dispersion con-

taining all of the soybean proteins would precipitate upon standing five to seven days at room temperature. The possibility of bacterial action was eliminated, since the coagulation took place even though the extracts were protected with toleuene and chloroform or after filtering through a Seitz filter and protecting against subsequent contamination. Microscopic examination, also, did not reveal the presence of any microorganisms. This coagulation was allowed to take place with several dispersions and, with only slight variation, 27% of the total nitrogen was observed to precipitate in each case. This value is in very close agreement with that obtained when extracts are heated at 75°C. for 5 hr. or less.

Experiments were made in an attempt to identify the precipitate. It was found that the cold precipitable fraction of the globulins was not affected by removal of this protein which precipitated at room temperature, and that, after its removal, no further protein could be precipitated by heating. The indication, then, is that the protein which precipitates at room temperature is the same as that which is coagulated by heating. Proof of this lies in the fact that electrophoresis patterns of an extract before and after precipitation at room temperature, as well as the pattern for the precipitate, showed that the proteins removed were the same as those precipitated by heating. The agglomeration, again, seemed to be the result of an interaction of the proteins (yielding an electrophoretically homogeneous complex). These experiments involving precipitation of aqueous extracts upon heating and at room temperature were also repeated with a 5% sodium chloride solution of the protein both before and after dialysis against sodium chloride. The results were practically identical with those obtained on water solutions. It should be pointed out that this agglomeration does not occur to a detectable degree at 4°C., and therefore does not alter the protein solutions during the four-day-dialysis period at that temperature in preparation for electrophoretic analysis.

Discussion

The Effect of Solvent Extraction of Meal. Although the results show that the greatest effect of cold alcohol extraction is on the globulin fraction of soybean protein, the other components are also altered to some extent by the treatment, because the loss of water extractability cannot entirely be accounted for by the decreased area of the globulin peak. Of particular interest is the observation that extraction with hot alcohol renders the globulin fraction almost completely nondispersible in water, whereas heating the proteins in an aqueous dispersion desolubilizes primarily those components other than globulins.

Apparently, the effect of heat is dependent upon the amount of water present.

On the basis of these experiments, the postulate (8) that alcohol extraction decreases water peptizability of the protein by removal of a natural peptizing agent would appear to be incorrect. If this were the only effect of alcohol extraction, it should still be possible to extract the globulin components with a sodium chloride solution, because these in native form are readily soluble in this solvent and do not require the presence of any other peptizing agent. The extractability of nitrogen from alcohol-treated meal with sodium chloride solutions, over and above that which is extractable with water alone, could not be demonstrated in this work. Thus, it seems that the primary effect of alcohol is to alter the globulin components in such a manner as radically to change their solubility properties.

The Effect of Heat. It must be emphasized that the formation of a single peak at the expense of several smaller peaks in the electrophoretic pattern of a mixture of soybean proteins is not unequivocal proof that interaction between the various component proteins has occurred. The possibility that each protein has been altered by the heat treatment so that all finally show the same electrophoretic mobility without undergoing interaction seems remote, however. Also, since the same phenomenon can apparently occur at room temperature, it would seem that it is not a result of the type of heat denaturation usually encountered in protein systems but that the heat treatment serves only to accelerate a denaturation reaction which is already able to proceed at room temperature.

Somewhat similar observations have been reported as resulting from the action of heat upon other protein mixtures in solution. Kleczkowski (6) found that on heating a mixture of the euglobulin and albumin fractions of normal rabbit serum the two proteins unite and form a complex. Van de Scheer, Wyckoff, and Clarke (10) have observed that a part of the protein of normal horse serum becomes denatured when warmed to 65°C., and a component is formed which exhibits an electrophoretic mobility approximately equal to that of β-globulin in the normal serum. This component arises initially at the expense of globulins but, when produced in amounts which approximate the total globulin present, incorporates considerable quantities of the albumin. The similarity between this observation and that noted upon heating a solution of the soybean proteins is noteworthy. The loss of some of the globulin fraction may be a result of its incorporation into the aggregate formed primarily by the interaction of the other proteins present in the soybean extract.

The analogy is inexact, however, in that it is the albumins of the soybean extract which undergo the primary aggregation while it is the globulin type proteins of blood serum which are the ones primarily involved when heated to 65°C. There is also the difference that the soybean albumins undergo the interaction at room temperature while the blood globulins apparently must be taken to a temperature where they become heat denatured before they show this tendency. As to the nature of the interaction, present data indicate that it involves an aggregation of primary proteins to form complexes which are still soluble or peptizable, but which contain molecules of the involved protein fractions in proportions which approximate the relative amounts of these fractions originally existent in the mixture. This is indicated by the fact that the observed mobility of the complex is always very close to the resultant mobility (m_R) which would be calculated from the relationship,

$$m_R = \frac{C_1m_1 + C_2m_2 + C_3m_3 \ldots}{C_1 + C_2 + C_3 \ldots}$$

where C_1, C_2, C_3, etc. represents the concentrations of the components in the uninteracted (original) mixture and m_1, m_2, m_3, etc. are their respective mobilities before interaction. In no case has there been any indication that the process involved in the formation of the complex is reversible.

The aggregation and subsequent precipitation of some of the soybean proteins, when allowed to stand in aqueous solution at room temperature, may offer an explanation for some of the changes which take place on storing soybeans. Jones and Gersdorff (5) have found that the storage of ground defatted soybean meal at room temperature resulted in a rather rapid decline in the amount of nitrogen that could be extracted by sodium chloride solutions. Although the experiments reported here involved protein solutions, it seems possible that the same type of desolubilization of protein as that which is observed to occur in solution at ordinary temperatures could occur also in stored meal because of the moisture which is present.

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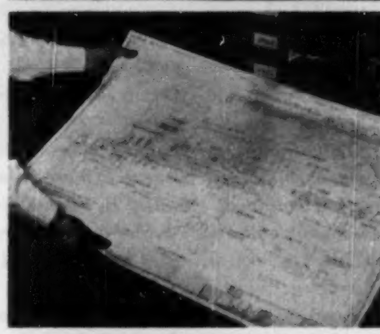

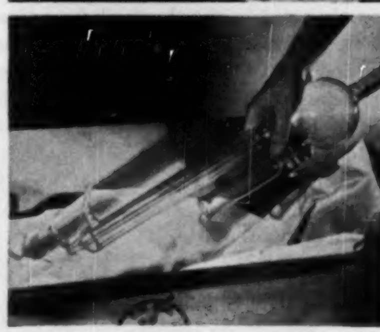

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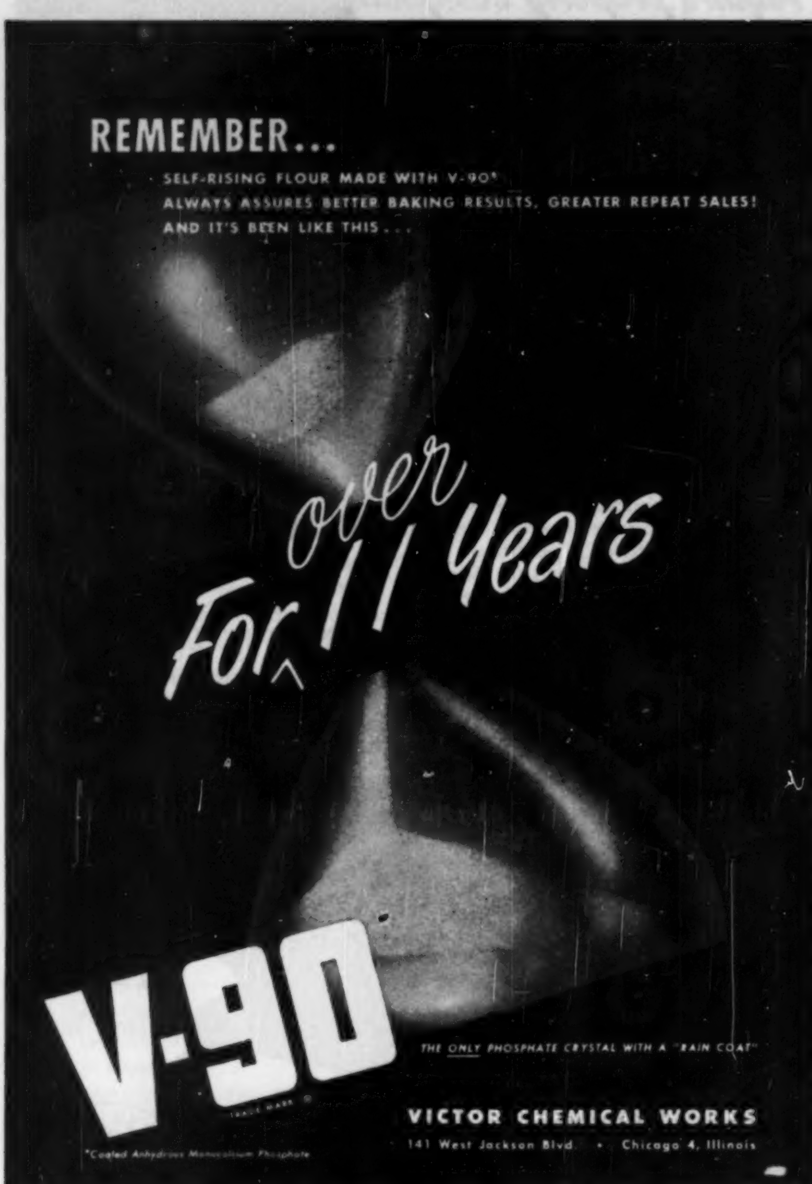
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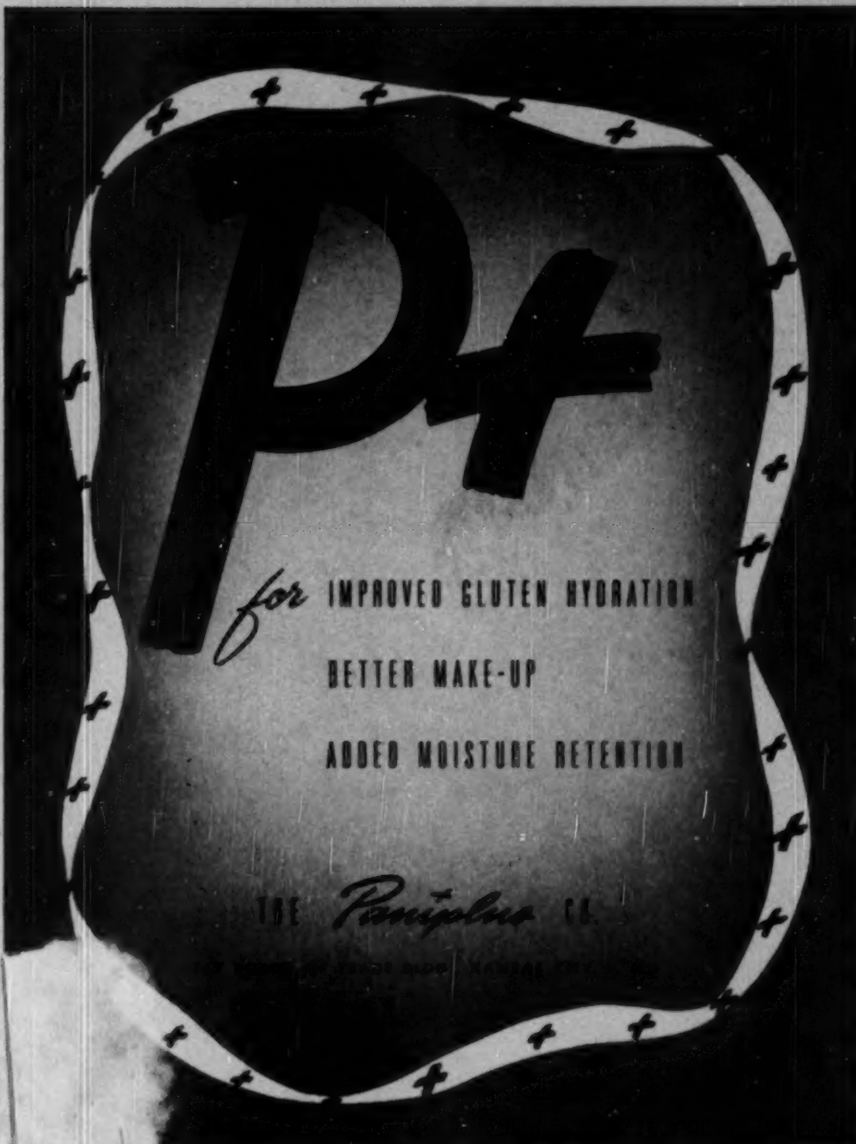
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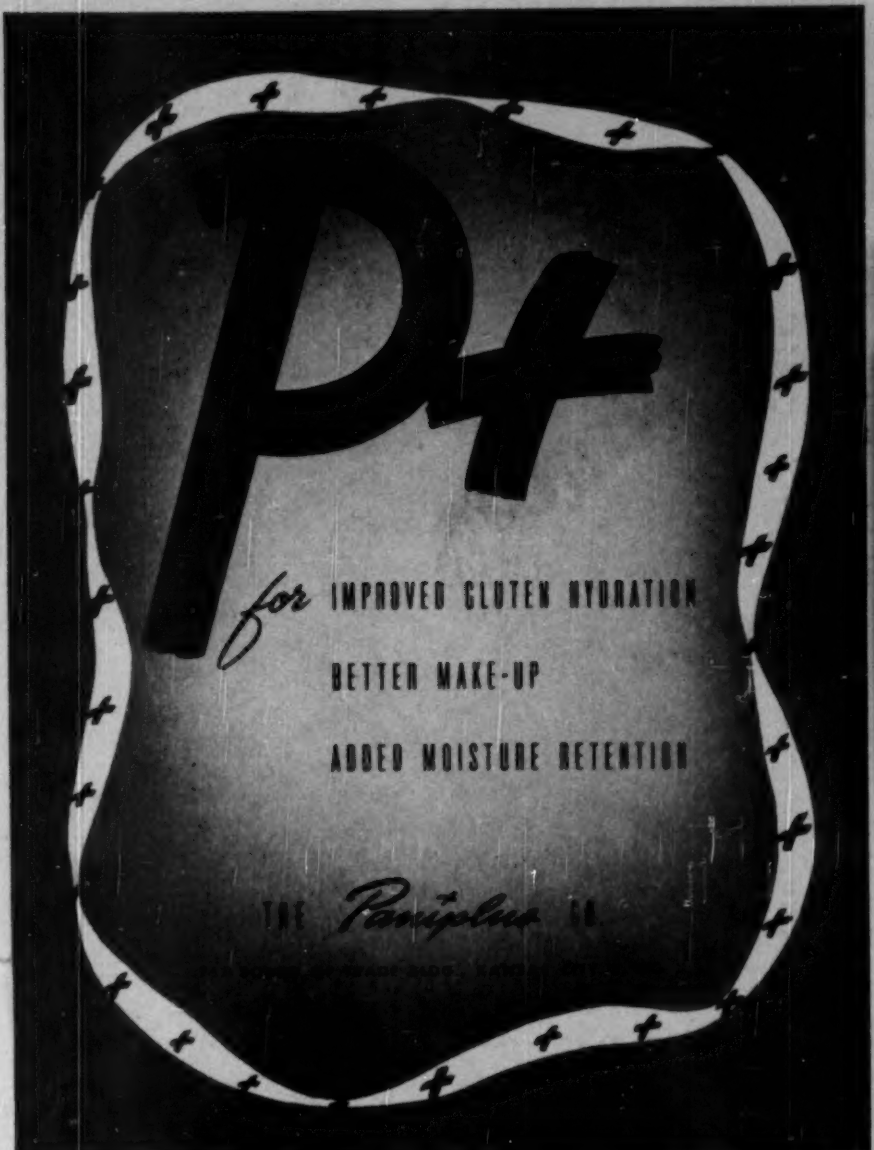
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